Early liver formation in zebrafish: a molecular and morphological approach

Emily Sarah Noël

The Division of Developmental Biology,

MRC National Institute for Medical Research,

UCL

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Abstract

The digestive tract and its accessory organs - liver, pancreas, and the inner lining of the swim bladder - are derived from the endodermal germ layer. In zebrafish the digestive system starts out as a rod at the embryonic midline, which subsequently becomes patterned and outgrowth of its associated organs occurs. Liver formation can be subdivided into three distinct phases; specification, budding, and differentiation and growth. Despite the importance of the liver for body homeostasis, relatively little is known about the mechanisms of liver development.

The transgenic line *Tg(gutGFP)*^{s854}, expressing GFP throughout the developing digestive tract, was used to identify the mutant lines *s436* and *4C1* in a forward genetic screen for mutants displaying defects in endodermal organogenesis. Initial analysis of both lines revealed a hypoplastic liver at 48 hpf, and in addition distinct defects in pancreas formation.

Positional cloning of the *s436* line identified a novel allele of *histone deacetylase 1* (*hdac1*), which plays distinct roles in endodermal organogenesis in zebrafish. Loss of Hdac1 causes defects in timely liver specification and subsequent differentiation, while mosaic analyses revealed a cell-autonomous role for Hdac1 in the endoderm in these processes. In addition, I have demonstrated that Hdac1 has specific functions in endocrine pancreas morphogenesis, as well as roles in exocrine pancreas specification and differentiation. Finally, loss of Hdac1 results in an expansion of the foregut endoderm in the domain from which the liver and pancreas originate, suggesting a scenario whereby Hdac1 may directly or indirectly restrict foregut fates while promoting hepatic and exocrine pancreatic specification and differentiation.

Phenotypic characterisation of endodermal organogenesis in *4C1* mutant embryos revealed a requirement for *4C1* in specification of the correct number of hepatic progenitors and hepatic bud morphogenesis. This is accompanied by a subsequent requirement for 4C1 in hepatic growth and duct morphogenesis. Additionally, *4C1* mutants exhibit defects in exocrine pancreas anlage outgrowth, leading to ectopic exocrine bud formation, suggesting a role for 4C1 in the morphogenesis of this organ. Preliminary analysis suggests that 4C1 may be required within the neighbouring tissue, the lateral plate mesoderm, to promote endodermal organogenesis.

Taken together, I have characterised two novel mutant lines, which despite initial phenotypic similarities, exhibit very distinct defects in liver as well as pancreas formation, highlighting important roles for these genes in the processes underlying endodermal organogenesis

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Declaration

I, Emily Sarah Noël, declare that the work presented in this thesis was performed in the laboratory of Dr. Elke Ober in the Division of Developmental Biology at the MRC National Institute for Medical Research (NIMR). I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the text. The work described in Appendix 1, isolation of zebrafish *Vitamin D binding protein,* was performed with the help of Zoya Arain, a Nuffield Bursary student. Figure 3.22, panels E and G, were contributed by Antonio Casal.

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Bmp	Bone morphogenetic protein
dpf	days post fertilisation
ECM	Extracellular matrix
ES cells	Embryonic stem cells
Fgf	Fibroblast growth factor
Hdac	Histone deacetylase
Hgf	Hepatocyte growth factor
hpf	hours post fertilisation
Hnf	Hepatocyte nuclear factor
LPM	lateral plate mesoderm
min	minutes
MO	morpholino oligonucleotides
RA	Retinoic Acid
RT	room temperature
SSLP	Simple sequence length polymorphism
TGF	Transforming Growth Factor
TSA	Trichostatin A
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter 1

Introduction

In the developing embryo, the digestive tract and its associated organs are derived from the endodermal germ layer. In zebrafish the endoderm forms at the blastoderm margin at around 4 hours post fertilisation (hpf; Ho and Kimmel, 1993; Warga and Nüsslein-Volhard, 1999) and at early somitogenesis stages comprises a sheet of cells situated ventrally in the embryo, in close contact with both the mesoderm and the yolk syncytial layer (YSL, Fig. 1.2). Subsequently, regionalisation of the endoderm occurs, and by 24 hpf the anterior endoderm comprises the pharyngeal and digestive tract endoderm, and the cells of the latter have migrated to the midline of the embryo, forming a multicellular rod constituting the immature digestive tract. Over the next 24 hours a subset of endodermal cells become specified to form the various organs associated with the digestive tract: the liver, pancreas and swim bladder (Ober et al., 2003).

The generation of multiple, complex structures such as the liver and pancreas from an initially homogeneous cell population requires the carefully controlled regulation of genes necessary for events such as cell-type specification and differentiation, balance of proliferation and apoptosis, as well as intricate morphogenetic changes. Zebrafish provide an ideal model organism to study the factors underlying hepatic organogenesis because, unlike in mammals, the embryonic liver is not the site of haematopoiesis, allowing independent analysis of the development of these two systems. In addition, small embryo size permits diffusion of oxygen to the major tissues in the body, allowing analysis of mutant embryos independent of circulation defects. Furthermore, endodermal organogenesis occurs within a relatively short time frame, while embryonic transparency allows easy visualisation of transgenic lines expressing fluorescent proteins throughout the digestive system. Forward genetic approaches have allowed the rapid generation of a

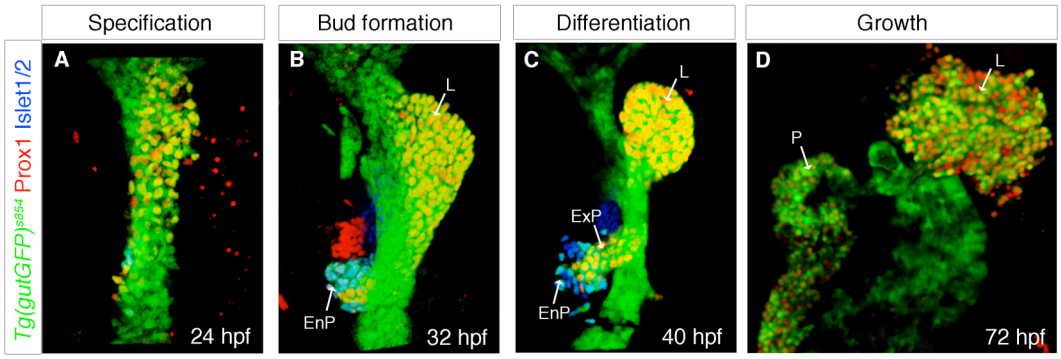


Figure 1.1 Time-course of liver organogenesis in zebrafish

Ventral projections of confocal stacks, anterior to top, using the *Tg(gutGFP)*^{s854} line to highlight the endoderm (green), stained with α-Prox1 in the hepatoblasts and exocrine pancreas (red), and α-Islet1/2 in the endocrine pancreas. Hepatoblast specification occurs by 24 hpf (A), followed by bud formation and outgrowth by 30 hpf (B). Subsequently, hepatic differentiation is initiated at 34 hpf (C) and growth of the liver begins 48 hpf (D). L, liver; EnP, endocrine pancreas, ExP, exocrine pancreas; P, combined pancreas.

wealth of zebrafish mutants. The fully sequenced zebrafish genome enables the isolation of the genomic position of the lesion underlying mutant phenotypes, allowing the identification of novel factors involved in endodermal organogenesis and the mechanisms by which they act, furthering our understanding of digestive system formation in vertebrates.

1.1 - Liver development

The morphological events of liver formation

Liver development in zebrafish can be divided into four distinct phases (Fig. 1.1, 1.2). The first phase comprises specification of hepatoblasts, the common progenitor of hepatocytes and bile duct cells. At around 14 hpf the endoderm forms a sheet-like structure (Fig. 1.2, A, B). The initial organisation of cells within the endoderm prior to hepatic specification is important in determining the presumptive fate of these cells. Fate mapping studies have demonstrated that at this stage, the cells that will give rise to the liver are situated most lateral, about 3 rows of cells away from the midline, while the cells forming the pancreas and intestine are predominantly found in more medial positions (Fig. 1.2, B C; Chung et al., 2008). Hepatoblast specific gene expression is present in the ventral foregut domain from around 22 hpf. After specification has occurred, and the endodermal cells have formed a multicellular rod at the embryonic midline, at 24 hpf the specified hepatoblasts are found in the ventral part of the endodermal rod at approximately the level of the second to third somite. The progenitors are part of an area referred to as the organ-forming endoderm, which comprises all endodermal cells marked by *Tg(gutGFP)*^{s854} expression from the anterior border of the hepatic domain to the posterior margin of the endocrine pancreas at 24-32 hpf or exocrine pancreas at 48 hpf (Fig.1.2). The first phase is

followed by liver bud formation. The nascent hepatoblasts aggregate anteriorly, and localise on the left side of the endoderm forming a small liver bud. Growth of the liver bud begins from around 30 hpf and is accompanied by leftward looping of the intestinal rod (Field et al., 2003b). This process is driven by the asymmetric migration of the bilateral lateral plate mesoderm (LPM), which neighbours the endoderm (Fig. 1.1, 1.2, 1.6; Horne-Badovinac et al., 2003). Subsequently, hepatic differentiation begins at around 34 hpf, and is marked by the onset of expression of differentiation markers in the liver. Concomitantly, a furrow begins to form between the budding liver and the adjacent digestive tract, ultimately leading to the formation of the extrahepatic duct, connecting the liver to the foregut. Finally, the liver grows rapidly from 48 hpf to 72 hpf, increasing in size but remaining roughly the same shape; from 72 hpf onwards the liver continues to grow and expands across the midline of the embryo (Field et al., 2003b), resulting in the formation of three distinct liver lobules in the adult zebrafish (Korzh et al., 2008).

Across vertebrate species, liver development is best understood in mouse. Similarly to zebrafish, initiation of liver development is accompanied by a thickening of the ventral foregut endoderm adjacent to the developing heart at E9 (Fig. 1.3). The subsequent steps of liver bud formation and growth differ between mouse and zebrafish. At E9.5 degradation of the basement membrane surrounding the presumptive liver bud is initiated, and the endodermal epithelium undergoes a transition to a pseudo-stratified epithelium (Bort et al., 2006; Sosa-Pineda et al., 2000). Subsequently, the hepatic endoderm undergoes full epithelial-mesenchymal transition, resulting in the cells of the presumptive liver delaminating and migrating into the surrounding septum transversum mesenchyme to form the liver bud in what

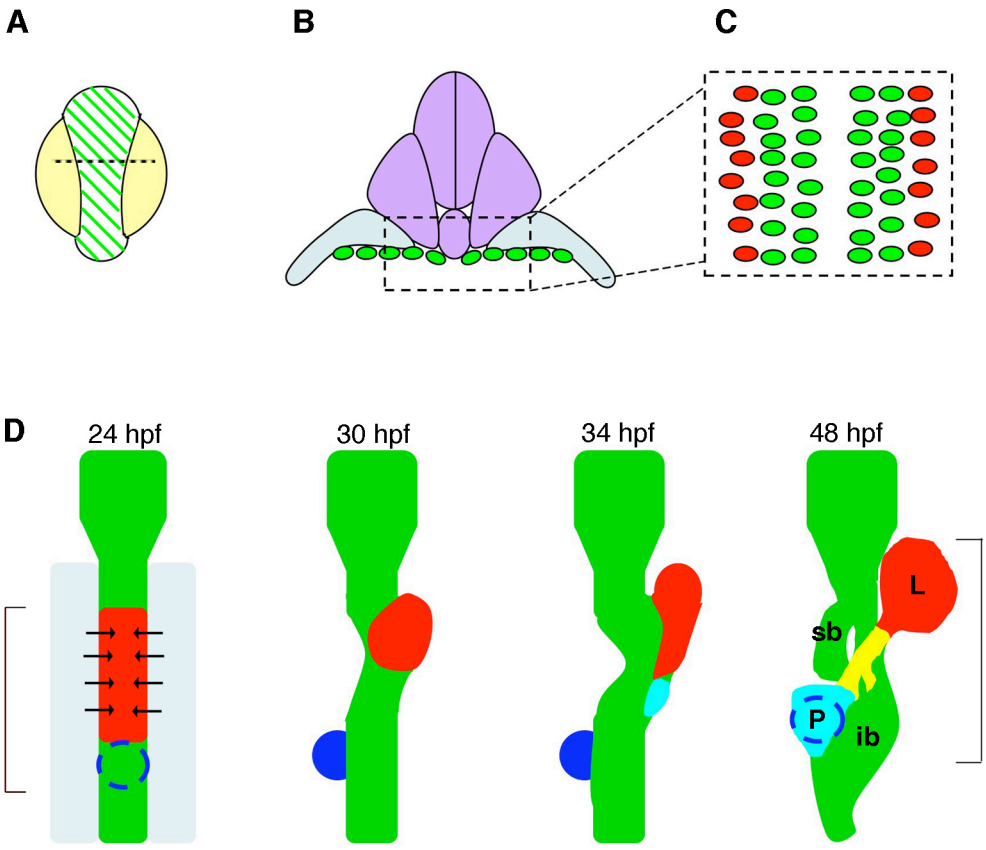


Figure 1.2 Liver organogenesis in zebrafish

(A-B) At 14 hpf the endoderm consists of a single sheet of cells situated ventrally in the embryo, dorsal to the yolk. (A) Dorsal view of a 14 hpf zebrafish embryo, green highlights the position of the endoderm. Dotted line indicates level of the transverse section in B. (B) Transverse section through a 14 hpf embryo. Green, endoderm; grey, LPM. (C) Organisation of cells within the endoderm at 14 hpf, ventral view. Endodermal cells in lateral-most columns will form liver (red), while cells within more medial columns contribute to the pancreas and digestive tract. (D) Time-course of liver organogenesis in zebrafish. Ventral views, anterior to top. Endoderm, green; liver, red; endocrine pancreas, blue; exocrine pancreas, cyan; extrahepatopancreatic duct, yellow; LPM, grey. At 24 hpf, the endoderm is a multicellular rod at the midline of the embryo. Signals from the neighbouring LPM specify hepatoblasts in the organ-forming region. At 30 hpf hepatoblasts have aggregated to form a liver bud, and the digestive tract has looped to the left. Around 34 hpf, a furrow begins to form between the liver and the adjacent intestine. By 48 hpf the liver and pancreas are connected to the adjacent foregut by the extrahepatopancreatic duct. Brackets in D indicate region termed organ-forming endoderm.

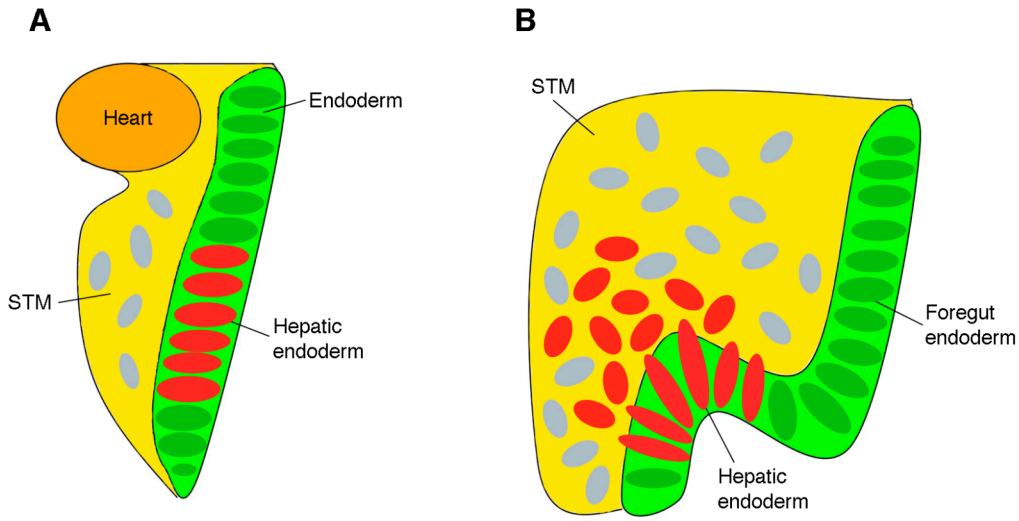


Figure 1.3 Hepatic organogenesis in mouse
At E9 the hepatic endoderm (red) lies adjacent to the septum transversum mesenchyme (STM; yellow/grey) (A). Once hepatoblast specification has occurred, heptoblasts delaminate and migrate into the surrounding mesenchyme, incorporating endothelial and mesenchymal cells to form the liver parenchyma from E9.5 (B). Green, endoderm; red, liver; yellow/grey, septum transversum mesencyme; orange, heart. Lateral views. (Adapted from Zaret et al., 2002)

has been described as 'hepatic cords' (Houssaint, 1980; Le Dourain, 1975; Medlock and Haar, 1983). In addition to endodermally derived hepatic cells, cells from the septum transversum mesenchyme become incorporated into the liver parenchyma once liver budding has begun. The liver bud subsequently grows at an accelerated rate, and hepatoblasts differentiate into either mature hepatocytes, the functional cells of the liver, or cholangiocytes, which will constitute the biliary system (Lemaigre, 2003). Despite the morphological differences in liver formation between mouse and zebrafish, these processes are regulated by similar molecular mechanisms.

Hepatic specification

Prior to hepatic specification, the endoderm is regionalised into the anterior foregut, and the posterior intestine. Regional patterning of the endoderm is mediated through Wnt and Fgf signalling. Repression of Wnt/β-catenin signalling in the anterior endoderm is required to promote foregut fates, while high levels of Wnt signalling in the posterior endoderm results in the inhibition of foregut fate in *X.laevis* and zebrafish (Dessimoz et al., 2006; Goessling et al., 2008; McLin et al., 2007). A similar role has been suggested for Fgf signalling in endoderm regionalisation in chick (Dessimoz et al., 2006). Once gut regionalisation has occurred, hepatic specification is initiated. Studies demonstrating the ability of foregut endodermal explants to express hepatic genes in the presence of mesoderm (Fukuda-Taira, 1981; Jung et al., 1999; Le Dourain, 1975; Zhang et al., 2004) suggested that interactions between these neighbouring tissues are critical for hepatic specification. The identification of key factors involved in the regulation of this process and key insights into the mechanisms by which they act in hepatic induction suggests that hepatoblast gene expression in ventral foregut endodermal cells occurs through a two-step model.

Firstly, the endoderm expresses factors that confer hepatic competence, allowing it to respond to hepatic induction signals, which are released from the neighbouring mesoderm in the second step.

Hepatic competence

The winged-helix domain containing *FoxA* transcription factors are expressed abundantly throughout the endoderm preceding hepatic specification and have been implicated in hepatic development (Costa et al., 1989; Lai et al., 1990; Odenthal and Nüsslein-Volhard, 1998). Loss-of-function studies in mouse have demonstrated that upon depletion of both *FoxA1* and *FoxA2,* the endoderm loses its competence to respond to inductive signals, highlighting combinatorial roles for multiple *FoxA* factors in provision of hepatic competence (Lee et al., 2005). Studies in mouse demonstrated that the *Albumin* enhancer contains FoxA1 binding sites that are essential for *Albumin* gene expression, the earliest definitive hepatic marker in mouse (Cascio and Zaret, 1991; Gualdi et al., 1996). These binding sites are occupied in both hepatic and non-hepatic tissue suggesting that FoxA factors expressed throughout the endoderm provide this tissue with the competence to respond to spatially restricted signals from the neighbouring tissues. Further studies have highlighted the mechanisms underlying this process. Cirillo and Zaret demonstrated that binding of FoxA1 opens the nucleosomal region of the DNA, resulting in an open chromatin conformation (Cirillo et al., 2002). Transcriptional repression and activation occur in part through regulation of chromatin conformation – closed chromatin conformations restricts access of transcription factors to the DNA, resulting in transcriptional repression. Conversely, an open chromatin conformation allows regulatory elements access to the DNA, and transcriptional activation can proceed.

Therefore, it is likely that binding of FoxA1 to the DNA allows the subsequent binding of regionally expressed transcription factors required for initiation of hepatoblast specification.

In addition, the binding of FoxA1 to the nucleosome has been demonstrated *in vitro* to facilitate the binding of another transcription factor: Gata4 (Cirillo and Zaret, 1999). *Gata4* is expressed throughout the definitive endoderm in mouse embryos before organ specification has occurred, and similarly to FoxA1, occupies the *Albumin* enhancer and is required for its expression (Bossard and Zaret, 1998). This suggests that these two genes act together to confer hepatic competence upon the endoderm by modifying chromatin conformation. The use of tetraploid embryo complementation to deplete *Gata4* or *Gata6* in the extraembryonic endoderm further confirmed that these genes are individually required to maintain *Albumin* expression *in vivo* (Duncan, 2005). Work in the frog *X. laevis* has demonstrated divergent roles for *Gata4* and *Gata5* in embryonic liver formation; *Gata5* is important for induction of liver specific genes, while *Gata4* is required in later stages of liver development, through interactions with *Gata6* (Haworth et al., 2008).

In zebrafish, single knockdown of *gata4* or *gata6* does not result in early hepatic defects. However, depletion of both these factors results in the complete absence of a liver bud (Holtzinger and Evans, 2005), suggesting that in zebrafish Gata4 and Gata6 act redundantly during early stages of liver formation to confer hepatic competence on the endoderm, driving hepatic specification. In addition, at later stages of development each gene appears to be individually required to promote growth of the liver (Holtzinger and Evans, 2005) demonstrating that different Gata factors exhibit distinct roles throughout organogenesis.

Altogether, studies in mouse, zebrafish and *X.laevis* have demonstrated that loss of individual Gata factors results in disruption of liver development, but not loss of the liver primordium (Haworth et al., 2008; Holtzinger and Evans, 2005; Reiter et al., 2001; Watt et al., 2007; Zhao et al., 2005). This indicates that these genes alone are not sufficient for providing hepatic competence, and that the function of additional genes such as FoxA factors are required for hepatic competence and specification. In this light, it is likely that FoxA factors play a more important role in providing the endoderm with hepatic competence.

Hepatic induction factors

Although the pan-endodermally expressed FoxA and Gata factors are required to confer hepatic competence upon a wide region of the endoderm, only a subset of these endodermal cells subsequently become specified as hepatoblasts. Explant and loss-of-function studies have demonstrated that this regional specification is likely the result of Fgf, Bmp and Wnt signals that originate from the neighbouring mesoderm (Chung et al., 2008; Jung et al., 1999; McLin et al., 2007; Ober et al., 2006; Rossi et al., 2001; Shin et al., 2007; Zhang et al., 2004).

Endodermal explants and ES cell studies have demonstrated that the cardiac mesoderm is capable of inducing the hepatic program in amniotes (Fukuda-Taira, 1981; Zhang et al., 2004). *Fgf1* and *Fgf2* are expressed in the cardiac mesoderm, and tissue explant studies in mouse have identified a role for these genes in hepatic specification in the endoderm (Jung et al., 1999), although depletion of both these genes in mouse results in viable animals (Miller et al., 2000). These signals are likely mediated through FGF receptors 1 and 4, which are expressed in the hepatic

endoderm (Jung et al., 1999). Subsequent studies outlining the mechanisms by which FGF mediates its effect on the endoderm has demonstrated that this induction occurs via the MAPK pathway (Calmont et al., 2006). In addition, in the absence of FGF signalling the underlying endoderm adopts a pancreatic fate (Deutsch et al., 2001). In this case, FGF signalling is hypothesised to regulate boundaries of *sonic hedgehog* (*shh*) expression, which is permissive to liver fate, but inhibits pancreatic fate (Deutsch et al., 2001). The use of transgenic zebrafish allowing temporal over-expression of a dominant negative form of *fgfr1* has demonstrated a genetically conserved role for Fgf signalling in hepatic induction in zebrafish, where Fgf signalling is required between 18 and 22 hpf to induce hepatic fate (Shin et al., 2007).

In addition to the requirement for Fgf signalling in hepatic induction, members of the Bmp signalling pathway have also been implicated in hepatic specification. Initial evidence for the involvement of BMP signalling in the promotion of hepatic fate in the endoderm arose from analysis of *Bmp4* depleted mice, where loss of *Bmp4* in the septum transversum mesenchyme resulted in morphological disruption of liver bud formation, and reduced expression of early hepatic genes (Rossi et al., 2001). Similarly, over-expression of dominant negative *bmpr* in zebrafish has demonstrated that Bmp signalling is required between 18 and 22 hpf for hepatic specification (Shin et al., 2007). The Bmp receptor Acvr1 transduces the Bmp signal in this specification process (Chung et al., 2008; Shin et al., 2007). Additionally, this study showed that the endoderm maintains its competence to respond to Bmp specification signals for at least 16 hours after specification occurs in wild type embryos. In zebrafish, *bmp2b* which is expressed in the LPM from around 10 somites is a good candidate to represent the Bmp ligand required in this process (Chung et al., 2008). Recent studies

have suggested that in zebrafish the liver and exocrine pancreas arise from a multipotent precursor (Chung et al., 2008). Overexpression of *bmp2b* directs presumptive digestive tract or pancreatic cells to adopt a hepatic fate (Chung et al., 2008). Similarly, induction of *Bmp4* expression in ES cells, or treatment of chick endodermal explants with Bmp2 heparin beads, results in initiation of hepatic gene expression (Gouon-Evans et al., 2006; Zhang et al., 2004). Interestingly, it was shown that loss of Bmp function results in a reduction of *gata4* and *gata6* expression in the hepatic endoderm (Shin et al., 2007). Depletion of these two *gata* factors in zebrafish results in failure to form a liver bud (Holtzinger and Evans, 2005). Furthermore, genetic analysis of the *Gata4* enhancer in mouse identified elements occupied by Fox and GATA factors that are essential for its expression and which are not occupied in *Bmp4* depleted mice (Rojas et al., 2005), together suggesting that Gata factors mediate the Bmp signal promoting hepatoblast formation.

The first demonstrated involvement of Wnt signalling in hepatic specification in zebrafish came from the identification in a forward genetic screen of the Wnt2b homologue *prometheus (prt/wnt2bb)*, which is expressed in the LPM from around 18 hpf onwards. (Ober et al., 2006). Loss of *prt/wnt2bb* results in transient absence of early hepatic gene expression and a severe reduction in liver size at 48 hpf, demonstrating the requirement for this gene in promoting hepatic specification. Furthermore, analyses of the zebrafish APC mutant displaying increased Wnt activity, and transgenic zebrafish expressing *wnt8* under the control of a heat shock promoter, have demonstrated that during early somitogenesis stages Wnt signalling is repressed to allow correct regionalisation of the endoderm, highlighting a biphasic requirement for Wnt signalling in early hepatic development (Goessling et al., 2008; Ober et al., 2006). This biphasic requirement for Wnt signalling integrates the requirement for

prt/wnt2bb in hepatic specification (Ober et al., 2006) with the reported requirement for repression of Wnt signalling at early stages in *Xenopus* development for liver formation (McLin et al., 2007). The role of Wnt signalling in hepatic induction is not well characterised in higher vertebrates, and candidates for mesodermally expressed Wnt ligands have not yet been identified. Alternatively, this may reflect a difference in the genetic requirements for hepatic specification in different species,

Currently, the epistatic relationships between Fgf, Bmp and Wnt signalling are poorly understood. In zebrafish, loss of Fgf or Bmp signalling does not result in a reduction of *prt/wnt2bb* expression in the mesoderm, however Bmp signalling can partially rescue the absence of Fgf signalling (Shin et al., 2007), suggesting crosstalk between these parallel pathways occurs during hepatic specification. Importantly, studies in zebrafish demonstrate that a common time window for Fgf, Bmp and Wnt requirements in hepatic specification between 18 and 22 hpf. While to date predominantly signalling pathways and transcription factors have been identified in hepatic induction, their interactions, regulations and mechanism of activity need to be further understood.

Liver bud formation

Once the competent endoderm has received hepatic induction signals from the adjacent mesoderm, hepatoblast gene expression is initiated. *Prox1* and *Haemopoietically expressed homebox* (*Hhex)* are two of the earliest genes known to be expressed in the nascent hepatoblast population in all vertebrates examined. Expression of both genes is initiated at around E8.5 in mouse, and 22 hpf in zebrafish. (Bogue et al., 2000; Keng et al., 1998; Field et al., 2003b; Yatskievych et al., 1999);

Burke and Oliver, 2002; Field et al., 2003b; Glasgow and Tomarev, 1998; Ober et al., 2006).

Functional analysis of *Hhex* knockout mice demonstrated its requirement in early liver formation and hepatoblast proliferation (Bort et al., 2004; Keng et al., 2000; Martinez Barbera et al., 2000). Further analysis revealed that liver development is arrested when the hepatoblasts delaminate and migrate into the surrounding mesenchyme, as the endoderm comprising the liver bud fails to undergo the transition to a pseudo-stratified epithelium, a process which is required for invasion of the hepatic cells in the surrounding mesenchyme and subsequent outgrowth of the liver (Bort et al., 2006). Loss of function analysis in zebrafish supports a conserved role for *hhex* in liver outgrowth (Wallace et al., 2001).

Hhex expression in the liver appears to be regulated by both competence and induction factors. FoxA1 and Gata4 have been demonstrated *in vitro* to transactivate the *Hhex* promoter (Denson et al., 2000; Rodriguez et al., 2001). In addition, analysis of the *Hhex* promoter in chick has identified Bmp response elements (Zhang et al., 2002), further strengthening the requirement for expression of genes such as FoxA1 within the endoderm to act in concert with signals such as Bmps from the overlying mesoderm in regulation of hepatic specification.

Similarly to *Hhex* deficient mice, *Prox1* knockout mice exhibit a reduction in liver size, and in addition the basal membrane surrounding the liver remains intact, and hepatoblasts fail to migrate into the surrounding mesenchyme (Burke and Oliver, 2002; Sosa-Pineda et al., 2000). *Prox1* overexpression *in vitro* revealed that Prox1 regulates the expression of ECM components (Papoutsi et al., 2007), supporting the role of Prox1 in liver morphogenesis and highlighting a mechanism for the regulation

of hepatoblast migration in mouse. Interestingly, this reflects the functional differences between the roles of *Hhex* and *Prox1* in bud formation, whereby *Hhex* is required for epithelial-mesenchymal transition, and *Prox1* is required for regulation of the ECM components important in hepatoblast migration. Moreover, *in vitro* studies suggest that both Prox1 and Hhex regulate hepatic proliferation (Kamiya et al., 2008) (Bort et al., 2004).

The continued expression of *Hhex* and *Prox1* during hepatic development suggests roles for these genes not only in the early proliferation and morphological changes associated with liver bud formation and outgrowth, but also in the differentiation programs initiated in hepatoblasts as they undergo the commitment to one of several cell types populating the adult liver. *Hhex* has been shown to interact with members of the HNF family to further stimulate its activity, suggesting a requirement for *Hhex* in maintenance of expression of genes required for hepatic differentiation (Tanaka et al., 2005), however direct targets of both *Hhex* and *Prox1* in hepatic development are yet to be determined in vertebrates.

Liver outgrowth and morphogenesis

Following specification and bud formation, the liver undergoes differentiation, changes in morphogenesis, and growth. In mouse, once hepatic specification has occurred, secondary interactions between the liver bud mesenchyme and the hepatoblasts are necessary to allow formation of the mature organ. The cells from the septum transversum mesenchyme, which contribute to the adult liver bud, are a major source of signals and transcription factors such as *Hlx* and *Lhx2* which are required to promote proliferation, survival and morphogenesis within the liver bud (Hentsch et al., 1996; Kolterud et al., 2004; Porter et al., 1997).

In zebrafish, although the early hepatic bud derives only from endodermal cells, the proximity of the liver to both the surrounding mesoderm and the YSL suggests that these tissues may interact with the adjacent endoderm to promote liver proliferation and morphogenesis.

A number of factors and signalling pathways have been implicated in these processes in different species, and the better understood ones are described below.

Fgf and Bmp signalling

In addition to playing important roles in hepatic specification, Fgfs and Bmps have been implicated in regulating the morphology and growth of the liver. Examination of the effects of Fgf signalling on murine hepatoblast cultures revealed roles for Fgf1, 4 and 8 in epithelial sheet formation, hepatic proliferation, and repression of duct formation (Jung et al., 1999; Sekhon et al., 2004). Similarly, activated Fgfr1 within the hepatic mesenchyme during liver outgrowth suggests a requirement for Fgf signalling in liver outgrowth (Sekhon et al., 2004). Analysis of *Bmp4* knockout mice, in addition to the use of Bmp antagonists in hepatic explant cultures has demonstrated a requirement for Bmp in the morphogenetic changes that occur in the liver after specification (Rossi et al., 2001). Additionally, disruption of *bmp2a* signalling from the LPM in zebrafish results in defects in hepatic proliferation (Huang et al., 2008), highlighting a requirement for Bmp signalling in hepatic growth. However, little is known about the mechanisms by which Fgfs and Bmps regulate morphogenetic changes and liver growth.

Hepatocyte growth factor and c-Met signalling

The identification of hepatocyte growth factor (Hgf) from the livers of human patients with hepatic failure initially suggested a role for this gene as a potent mitogen, playing an important role in liver regeneration in adults (Gohda et al., 1988; Gohda et al., 1986; Weidner et al., 1991). In mouse, Hgf is expressed in the septum transversum mesenchyme, and the hepatoblasts of the liver bud respond to Hgf signals through the tyrosine kinase receptor c-Met to upregulate DNA synthesis (Defrances et al., 1992). Depletion of *Hgf* or *C-met* in mouse leads to liver hypoplasia due to reduced levels of proliferation and increased apoptosis (Bladt et al., 1995; Schmidt et al., 1995). In addition, *c-Met* null ES cells cannot contribute to the liver, likely due to an inability to respond to Hgf signalling (Schmidt et al., 1995), further supporting the role of Hgf/*c-Met* signalling in hepatic outgrowth.

Wnt/β-catenin signalling

A number of Wnt ligands, such as *Wnt3A* and *Wnt1*, as well as high levels of activated β-catenin, are found in the liver and their individual roles in different aspects of hepatic development are emerging (Monga et al., 2002; Monga et al., 2003; Suksaweang et al., 2004; Thompson and Monga, 2007). The use of pan-endodermal and hepatoblast specific Cre lines has revealed dynamic temporal requirements for Wnt signalling in hepatic development. Early hepatoblast depletion of β-catenin results in disrupted hepatic morphology, and increased levels of apoptosis (Tan et al., 2008), following the reported requirements for Wnt signalling in hepatic induction (Goessling et al., 2008; Ober et al., 2006). Altering Wnt signalling at a later stage, by conditional hepatoblast-specific APC knockout resulting in upregulated β-catenin, led to increased formation of ductal structures and liver hypoplasia (Decaens et al., 2008). Accordingly, over expression of Wnt3A or depletion of β-catenin also led to the loss

of biliary markers, suggesting that β-catenin is involved in hepatocyte and biliary lineage segregation, in addition to the development of the biliary epithelium (Hussain et al., 2004; Micsenyi et al., 2004). *Ex vivo* hepatocytes cultured with Wnt3A, overexpression of *wnt8* in zebrafish, or overexpression of β-catenin in chick embryos resulted in liver hyperplasia (Hussain et al., 2004; Suksaweang et al., 2004) (Goessling et al., 2008). Conversely, knockdown of β-catenin resulted in decreased proliferation and increased apoptosis (Micsenyi et al., 2004), thus suggesting that canonical Wnt signalling is required to promote proliferation and cell-survival in the liver. Taken together, this suggests that β-catenin is required for segregation of the hepatic and biliary lineages, as well as highlighting roles for Wnt/β-catenin signalling in promoting proliferation and cell survival within a differentiated hepatocyte population. In addition, Wnt ligands appear to act together with other factors such as HGF to regulate β-catenin stabilisation (Monga et al., 2002) and modulate liver growth.

Transforming growth factor-β signalling

During hepatic organogenesis the liver bud mesenchyme expresses multiple Transforming Growth Factor β (TGFβ) ligands (Pelton et al., 1991), as well as various TGFβ receptors, including TGFβRIII (Stenvers et al., 2003). TGFβRIII depleted mice exhibit a hypoplastic liver due to defects in proliferation and increased levels of apoptosis, suggesting a role for TGFβ signalling in proliferation and survival in the liver (Stenvers et al., 2003).

TGFβ signalling is primarily mediated by the downstream effectors Smad2 and Smad3, both of which have been shown to be important for liver development (Ju et al., 2006). Mice lacking one copy each of *Smad2* and *Smad3*, or that are depleted of

the Smad2/3 interacting protein *Elf,* exhibit liver hypoplasia despite no apparent defects in liver marker expression (Tang et al., 2003). It has been suggested that this hypoplasia results from impaired hepatocyte migration due to reduced levels of β-integrin, which controls the response of hepatocytes to the extracelluar matrix (Weinstein et al., 2001). Concomitantly, *in vitro* studies have demonstrated a role for TGFβ signalling in controlling directional migration of hepatocytes by modulation of the ECM component Fibronectin (Binamé et al., 2008)

Cell cycle regulation and hepatoblast survival

Progressive growth of the liver during development requires regulation of the cell cycle and promotion of proliferation. The characterisation of zebrafish mutant lines generated in forward genetic screens has significantly contributed to our knowledge of cell cycle regulation in hepatic development. The *pescadillo* mutant was identified in such a screen, and exhibits defects in outgrowth of the liver from around 3 dpf onwards, (Allende et al., 1996). *Pescadillo* encodes a BRCT domain containing protein which is expressed widely throughout the embryo at early stages of development, however, by 48 hpf become restricted to highly proliferative regions such as the liver, pancreas and gut, as well as the ganglion cell layer in the eye and the ventral forebrain (Allende et al., 1996). Supporting this, studies in mouse demonstrated an upregulation of *Pescadillo* as hepatocytes enter the cell cycle (Lerch-Gaggl et al., 2002).

During early stages of liver growth zebrafish embryos express *uhrf1*, encoding a ubiquitin like protein required for cell-cycle progression *in vitro*, at high levels in the liver from 54 hpf when the liver is rapidly increasing in size. *Uhrf1* mutants exhibit liver hypoplasia due to proliferation defects, however patterning and differentiation of

the liver is unaffected suggesting that *uhrf1* is required only for growth of the liver (Sadler et al., 2007). Further, *liver-enriched gene* (*leg1) 1*, identified in a microarray for liver enriched genes, is expressed in the liver from 2 dpf onwards and is required for expansion of the liver once budding has occurred (Cheng et al., 2006). From 3 dpf onwards *digestive expansion factor (def)*, is expressed in the digestive system and similarly to *uhrf1* mutants, *def* mutants exhibit defects in growth, but not differentiation of the liver (Chen et al., 2005).

In addition to the identification of factors required for cell cycle regulation, several genes have been identified that are implicated in regulation of apoptosis. Overexpression of the Bcl-related gene *zfBLP1* and *zfMcl-1A* in zebrafish results in liver hyperplasia at 5 dpf, due to elevated levels of proliferation. Interestingly, levels of apoptosis are also increased in these embryos, however are insufficient to maintain normal liver size, suggesting that these genes may be involved in regulating the fine homeostatic balance between proliferation and apoptosis required for liver growth and regulation of adult liver size.

Hepatocyte differentiation

Once the liver bud is established, growth of the liver is initiated and bipotent hepatoblasts undergo differentiation in either hepatocytes forming the functioning units of the liver, or biliary epithelial cells, which make up the intrahepatic biliary system.

Hepatic differentiation begins around E17 in mouse, and 34 hpf in zebrafish, and is marked by the onset of the expression of genes such as *C/ebpα, C/ebpβ* and *Hnf4* in mouse (Birkenmeier et al., 1989; Sladek et al., 1990), and *ceruloplasmin* and *selenoprotein Pb* in zebrafish (Korzh et al., 2001; Thisse et al., 2003). Subsequently,

the onset of expression of functional differentiation markers such as *liver fatty acid binding protein (lfabp)* and *transferrin*, from around 3 dpf in zebrafish, is accompanied by adoption of a cuboidal morphology and the formation of bile canaliculi, connecting the hepatocytes to the biliary system (Korzh et al., 2008; Mudumana et al., 2004).

C/ebp encodes a CCAAT enhancer binding protein, a transcription factor important for regulating many genes in the fetal liver and other organs. Depletion of C/EBPα in mouse results in upregulation of proliferation and neonatal lethality due to hypoglycaemia suggesting that this gene is required to promote proliferation and functional differentiation of the liver (Wang et al., 1995). Supporting this, *ex vivo* studies have demonstrated that C/EBPα facilitates HGF-mediated *Albumin* expression and hepatocyte proliferation (Suzuki et al., 2003). In addition, C/EBPα depletion results in increased hepatic expression of HNF6, and subsequent biliary cell differentiation, suggesting that not only does C/EBPα suppress proliferation and promote cell cycle exit and differentiation, but also acts to suppress formation of the biliary system (Yamasaki et al., 2006).

In addition to a requirement for C/EBPα in hepatocyte differentiation, various hepatocyte nuclear family (HNF) members have been implicated in different aspects of hepatic and biliary differentiation, of which the role of *Hnf4α* is best understood. In zebrafish embryos *hnf4α* is expressed in the liver from 48 hpf onwards (Field et al., 2003b; Kudoh et al., 2001) and in mouse, *Hnf4α* is expressed in hepatoblasts from E9. Conditional inactivation of *Hnf4*α in mouse liver results in perturbed hepatic architecture, disrupted cell contacts, and a failure to accumulate hepatic glycogen (Parviz et al., 2003), suggesting roles for *Hnf4*α in the morphogenesis of the liver, and

control of glucose metabolism. Overexpression of *Hnf*4α in hepatoma cell lines results in the expression of epithelial marker genes (Späth and Weiss, 1998) supporting the requirement for *Hnf4*α in regulating the mesenchymal-epithelial transitions in the differentiating liver. *in vitro* studies using HNF4α null cells demonstrate that upon transplantation in murine livers in a regeneration model these cells are capable of forming only bile ducts and not hepatocytes (Hayhurst et al., 2008), suggesting that similar to C/EBPα, HNF4α may be responsible for promoting hepatocyte differentiation or repressing bile duct formation in hepatoblasts.

Microarray analysis of HNF binding sites in liver enriched genes in human and zebrafish revealed high numbers of genes potentially regulated by HNF family members (Cheng et al., 2006; Odom et al., 2004). Interestingly, in human the most common HNF binding site was HNF4, which was predicted to bind to slightly under half the liver enriched genes (Odom et al., 2004). However, in zebrafish 90% of liver enriched genes had HNF6 binding sites (Cheng et al., 2006), highlighting evolutionary differences in the roles of various HNF family members in hepatic and biliary development.

Unlike in zebrafish, in mouse the liver is the site of embryonic haematopoiesis. Haematopoietic stem cells migrate into the fetal liver, where they produce daughter haematopoietic cells before haematopoiesis is established in the bone marrow at later stages. The cytokine Oncostatin M (OSM) is produced by haematopoietic cells in the fetal liver, and *in vitro* studies indicate that this factor is able to drive the differentiation of hepatocytes (Miyajima et al., 2000). Further functional studies indicate that while both OSM and HGF are capable of inducing *Albumin* expression, only OSM can induce Albumin secretion (Kamiya et al., 2001) suggesting that these

two genes are involved in different pathways regulating hepatocyte maturation. Due to the absence of haematopoietic cells in the liver of zebrafish embryos it remains to be determined whether OSM is produced by alternative cells and whether this gene is required for hepatic development in zebrafish.

While many transcription factors have been implicated in various aspects of hepatic development, relatively little is known about the mechanisms regulating the interactions between transcription factors and their DNA targets, as well as RNA processing. Interestingly, recent work has revealed the requirement for chromatin remodelling factors in regulating hepatic differentiation and maturation. Knockdown of *dnmt2*, a DNA methyltransferase, results in a loss of *lfabp* and *transferrin* expression, although liver size is only mildly affected (Rai et al., 2007). Notably, this phenotype is specific to Dnmt2, as loss of Dnmt1 function resulted in defects in terminal differentiation of the intestine and exocrine pancreas, but not the liver (Rai et al., 2006), highlighting a mechanism whereby different chromatin modifying factors may play very specific roles in the differentiation of individual endodermal organs.
Furthermore, *nil per os* (*npo*) is expressed in the digestive tract and associated organs from around 48 hpf onwards, and encodes a protein similar to the yeast protein Mrd1p, an RNA recognition protein required for pre-ribosomal RNA processing. *Npo* mutants exhibit liver hypoplasia, in addition to defects in expression of cytokeratin in the liver and failure to form a functioning biliary system, suggesting that *npo* is required for mediating late growth and cytodifferentiation of the liver (Mayer and Fishman, 2003). Together, this demonstrates the requirement for transcription factors and signalling molecules, as well as DNA and RNA remodelling factors in regulating

hepatic differentiation. However, further work needs to be undertaken to understand the mechanisms by which these genes act to drive functional hepatic differentiation.

Biliary system formation

In the mature liver the biliary system forms an intricate network facilitating hepatic function and bile secretion. During development, concomitantly with hepatocyte differentiation, the biliary system forms, and consists of the intrahepatic ducts, which via the extrahepatic duct connect the liver to the gall bladder and adjacent digestive tract. This requires carefully controlled gene expression allowing commitment of bipotential hepatoblasts to either the hepatic or biliary lineage.

Duct formation in zebrafish has been characterised using antibodies such as 2F11 and Alcam, which are expressed in both intra- and extrahepatic biliary epithelia (Dong et al., 2007; Sakaguchi et al., 2008). From around 40 hpf hepatoblasts express 2F11 ubiquitously, and at around 50 hpf 2F11 and Alcam are expressed throughout the liver, localised at hepatocyte cell membranes (Sakaguchi et al., 2008). Over the next 30 hours, the hepatocytes become polarised and endothelial cells invade the liver along the future basal membrane of the hepatocytes. The basal invasion of endothelial cells results in 2F11 and Alcam localisation to the apical surfaces of some of the hepatocytes, ultimately resulting in the differentiation and refinement of bipotential hepatoblasts into biliary epithelia (Sakaguchi et al., 2008).

In addition to important roles during early stages of hepatic development, the Onecut (HNF) family of transcription factors have been implicated in both mouse and zebrafish to be required for development and elaboration of the intrahepatic biliary system. Two members of the onecut family have been identified in zebrafish: *onecut1/hnf6* and *onecut3* (Matthews et al., 2008; Matthews et al., 2004). In contrast,

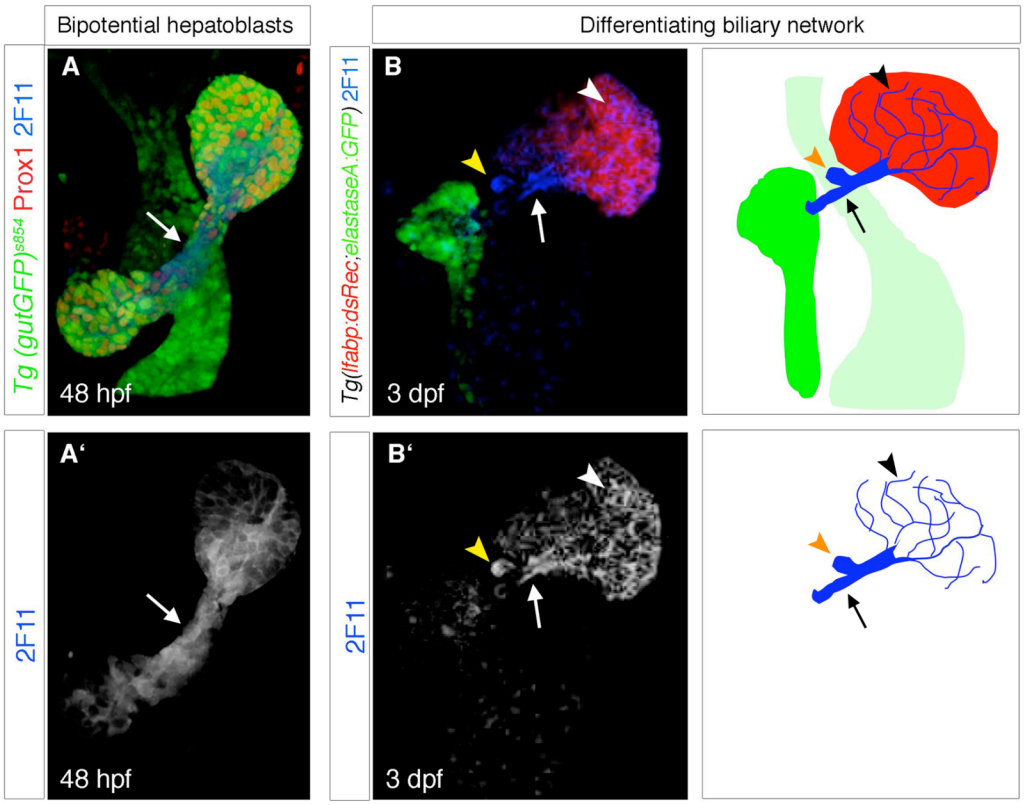


Figure 1.4 Biliary system formation in zebrafish

(A-B') Ventral projections of confocal stacks using the *Tg(gutGFP)*^{s854} line, stained for Prox1-positive liver and exocrine pancreas (red) and 2F11-positive duct tissue (blue, A) or the *Tg(lfabp:dsRed;elastaseA:GFP)* line stained for 2F11 (B). At 48 hpf 2F11 expression is heightened in the extrahepatic duct, and expressed at low levels in the bipotent hepatoblasts (A). At 3 dpf 2F11 remains heightened in the extrahepatic duct, and within the liver becomes restricted to the differentiating biliary network (B). White arrow, extrahepatic duct; white arrowhead, intrahepatic ducts; yellow arrowhead, gall bladder. (C) Schematic representation of the liver, pancreas and ductal system shown in (B). Red; liver, blue; ductal system, green; pancreas. Blach arrowheads denote intrahepatic ducts, black arrow highlights the common extrahepatopancreatic duct, orange arrowhead depicts gall bladder.

three are known to exist in mammals: Onecut1, 2, and 3, all of which are expressed in the liver during development (Hong et al., 2002; Matthews et al., 2004). Knockout studies have begun to uncover the epistatic relationship and roles of these genes in formation and elaboration of the biliary network. Morpholino-mediated knockdown of *onecut3* in zebrafish results in severe disruption of the biliary system, characterised by a reduction in number of intrahepatic bile ducts (Matthews et al., 2008). Loss of *Onecut1/hnf6* in mouse and zebrafish results in disruption of the biliary system, characterised by perturbed development of the intrahepatic ducts (Matthews et al., 2004). Although knockdown of both *onecut1* and *onecut3* results in biliary disruption, the phenotypes are quantitatively different, and studies have revealed that the epistatic relationship between *Onecut1* and *Onecut3* differs between zebrafish and mouse. The biliary phenotypes observed in *onecut1* zebrafish morphants are less severe than those observed in *onecut3* morphants, and knockdown of *onecut3* results in reduced expression *onecut1* demonstrating that *onecut3* is acting upstream of *onecut1* in regulating intrahepatic biliary development in zebrafish. Conversely, in mouse Onecut1 can bind to and induce expression of *Onecut3* (Pierreux et al., 2004), suggesting that *Onecut3* is downstream of *Onecut1* in mouse, and therefore that zebrafish *onecut3* is the functional orthologue of mammalian *Onecut1* in directing initial elaboration of the intrahepatic biliary netowrk. Furthermore, knockdown of *onecut1* in zebrafish is accompanied by a downregulation of *Vhnf1/Hnf1β* (Matthews et al., 2004), and in mouse loss of *Hnf1β* induces a similar phenotype to that of *Onecut1* depletion (Coffinier et al., 2002) demonstrating that Onecut1 acts through *vhnf1/hnf1β* to direct elaboration of the biliary tree in both zebrafish and mouse. Further, in mammals Onecut1 does not regulate the expression of *Onecut2,* and depletion of Onecut2 results in biliary defects slightly different from those observed

in *Onecut1* null mice (Clotman et al., 2005) demonstrating independent requirements for Onecut1 and Onecut2 in biliary formation in mouse.

The mechanisms by which these factors exhert their effect on segregation of the hepatic and biliary lineages are beginning to emerge. Clotman and colleagues used loss of function mice to show that both Onecut1 and Onecut2 act in concert to modulate a gradient of TGFβ signalling, with high levels of TGFβ activity observed near the portal vein for biliary cell differentiation and inhibition of hepatocyte differentiation, suggesting that TGFβ/Activin signalling is required for biliary cell differentiation (Ader et al., 2006; Clotman et al., 2005). Importantly, analysis of *Hhex* conditional knockout mice revealed a requirement for *Hhex* in differentiation of hepatoblasts into the biliary lineage, and in regulating expression of early biliary factors such as *Onecut1* and *Hnf1β* (Hunter et al., 2007).

The Notch pathway has been implicated to act in parallel to the Onecut pathway in regulating biliary system formation. Human mutations in the ligand JAGGED1 result in Alagille syndrome, characterised by paucity of inter-lobular bile ducts. Further, characterisation of *Jagged1* depleted mice revealed phenotypic similarity to Alagille syndrome, and double heterozygotes for *Jagged1* and *Notch2* mutations generate a more representative model. Subsequent analysis of *Jagged*1 and *Notch2* expression in mouse revealed an expression pattern that correlated with their involvement in bile duct formation (Loomes et al., 2002; McCright et al., 2002) suggesting that these genes interact in formation of the biliary system. Similarly, functional studies in zebrafish confirmed that Jagged-mediated Notch signalling is required for biliary system formation, identifying roles for a number of Jagged ligands (*jagged2* and *jagged3*) and Notch receptors (*notch2* and *notch5*; Lorent et al., 2004). The examination of hepatocyte cultures transfected with Notch resulted in upregulation of

biliary marker *Hnf1β,* suggesting this gene is a common mediator of both the Notch and Onecut pathways in biliary development. Conversely, inhibition of Notch signalling promoted hepatocyte differentiation marker expression (Tanimizu and Miyajima, 2004). Altogether these data suggests a conserved role in zebrafish and mouse for Notch signalling in promoting biliary differentiation and morphogenesis.

In addition to well characterised roles for *Onecut* factors and Notch signalling in biliary development, Wnts, Fgfs and Bmps have all been implicated in the segregation of biliary and hepatocyte lineages. Knockdown of APC, resulting in upregulation of β-catenin in mouse, leads to increased differentiation of intrahepatic bile ducts (Decaens et al., 2008), suggesting a role for canonical Wnt signalling in differentiation of the biliary system. Recent work in chick has demonstrated that Fgf receptors and *Bmp4* are expressed in the intrahepatic bile ducts and hepatic mesenchyme respectively (Yanai et al., 2008). Explant cultures identified that Fgf7 and bFgf can induce biliary epithelial cell differentiation, and that this effect is enhanced by Bmp4 and the presence of ECM components (Yanai et al., 2008), highlighting additional roles for Fgf and Bmp signalling in promotion of biliary differentiation. However, the genetic interactions between these and the *Onecut* and Notch pathways is unknown.

The extrahepatic duct appears to originate from the foregut adjacent to the forming organs. During its formation in zebrafish 2F11 becomes heightened in the extrahepatic duct connecting the liver and pancreas, along with a downregulation of Prox1 expression. *fgf10* is expressed in the mesenchyme surrounding the developing extrahepatic duct from around 56 hpf onwards. In *fgf10* mutants Prox1 is ectopically expressed in the extrahepatopancreatic duct and the expression of hepatocyte markers

is expanded into this region (Dong et al., 2007), suggesting that Fgf10 is required to promote extrahepatic duct formation and maintain its fate by inhibiting hepatic and pancreatic differentiation in multipotent endodermal progenitors. Additionally, depletion of *Hes1* in mice leads to a similar phenotype resulting in ectopic pancreas specification at the expense of the extrahepatopancreatic duct (Sumazaki et al., 2004), implicating Notch signalling formation of the extrahepatic biliary system in addition to its well characterised role in intrahepatic biliary differentiation.

The role of the vasculature in liver development

In the mature liver, the blood vessels and bile ducts form an intricate network to mediate blood flow and bile secretion through the organ. In both zebrafish and mice the organisation of these two networks is similar – mature hepatocytes form a single layer of cells which is flanked on one side by an intrahepatic bile duct, and on the other side by an artery (Fig. 1.5), resulting in optimal liver function.

In mouse, prior to liver bud outgrowth, endothelial cells surround the newly specified hepatic endoderm, and during bud formation become incorporated into the liver parenchyma. In the absence of endothelial cells hepatic specification occurs but is arrested before liver outgrowth begins, suggesting endothelial cells are required for hepatoblast delamination and migration (Matsumoto et al., 2001). Subsequently, during hepatic organogenesis endothelial cells surround the liver from around 36 hpf, and begin to invade the liver from around 55 hpf (Sakaguchi et al., 2008). *Cloche* mutants lack endothelial cells and exhibit no defects in liver bud formation at 48 hpf, suggesting that endothelial cells are not required for early stages of hepatogenesis (Field et al., 2003b). However at later stages, in the absence of endothelial cells and

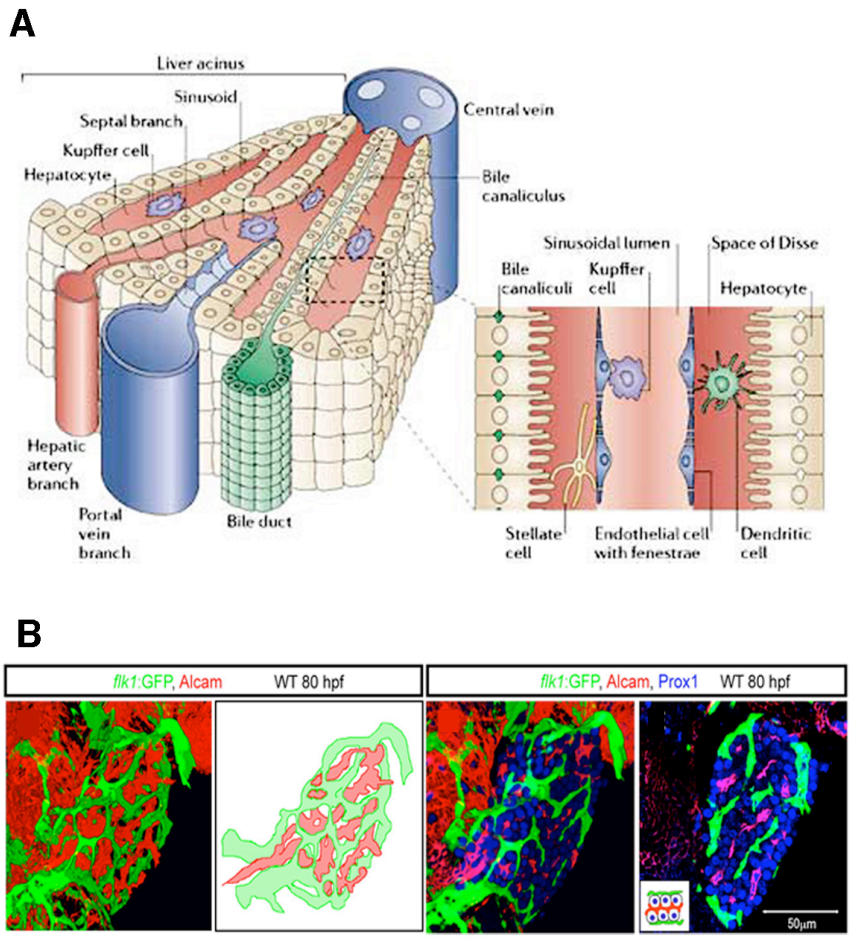


Figure 1.5 Biliary and vascular organisation in the liver

(A) Organisation of the biliary and vascular networks in a mouse liver lobule. Hepatocytes are organised as a single layer between the sinusoids, connecting the central vein to the portal vein and hepatic artery, and the bile duct. Bile canaliculi form between adjacent hepatocytes. Adapted from (Adams and Eksteen, 2006).

(B) Organisation of the biliary and vasulcar networks in the zebrafish liver at 80 hpf. The vascular network, highlighted by *flk1* GFP (green) is closely intertwined with the biliary network, highlighted by Alcam (red). Sections through the liver, with Prox1 (blue) expressed in hepatocytes, demonstrates a similar organisation to that observed in mice, with hepatocytes forming a single layer of cells between the vascular and biliary networks. Adapted from (Sakaguchi et al., 2008).

liver vascularisation, the liver fails to grow after around 55 hpf (Korzh et al., 2008). Based on these studies it has been proposed that three stages of liver growth are observed – until 55 hpf the liver undergoes endothelial cell independent growth; 55 hpf to 72 hpf comprises a phase of endothelium dependent growth, and from 72 hpf onwards the accelerated growth of the liver is the result of circulation dependent growth (Korzh et al., 2008).

As described earlier, endothelial cell invasion of the liver in zebrafish is required for timely formation and elaboration of the intrahepatic biliary system. Interestingly, knockout studies in mouse have suggested the converse: that formation of the biliary system is required for correct vascularisation of the liver (Clotman et al., 2003). These differences may result from alternative mechanisms of liver vascularisation in mouse and zebrafish.

The LPM and digestive tract morphogenesis

In addition to requirements in hepatic specification, LPM differentiation drives additional processes in zebrafish. Morphological analysis of digestive tract formation in zebrafish reveals that between 24 hpf and 30 hpf the digestive tract loops to the left side of the embryo at the level of the budding liver (Field et al., 2003b). Analysis of transverse sections of zebrafish embryos at 24 hpf revealed that the LPM represents bilateral unpolarised epithelia, each comprised of two sheets in a U-shape, situated lateral to the endodermal rod. Over the next 6 hours, these sheets become polarised, so that the side of the U nearest the liver is composed of columnar cells, and the side of the U situated away from the endoderm is composed of squamous cells. Concomitant with this polarisation, the lateral plates migrate asymmetrically. The left lateral plate migrates dorsally to the endoderm and budding liver, while the right

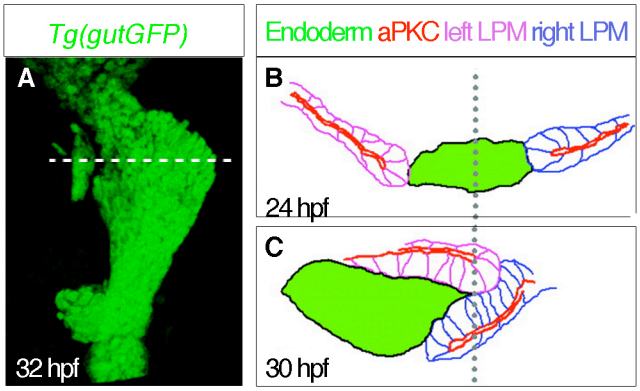


Figure 1.6 Asymmetric LPM migration directs digestive tract looping in zebrafish

(A) The digestive tract, visualised using the *Tg(gutGFP)*^{s854} line, which is situated at the embryonic midline at 24 hpf, loops to the left of the midline at 30 hpf at the level of the budding liver. (B, C) Schematic representation of transverse sections through the organ-forming endoderm at the level indicated in A. At 24 hpf the midline endoderm is flanked by the bilateral unpolarised LPM epithelia (B). At 30 hpf the left LPM has migrated dorsal to the endoderm, and the right LPM has migrated ventro-laterally (C), driving the leftward looping of the digestive tract observed in A. The LPM has become polarised, each sheet comprising a bilayer of squamous and epithelial cells (C). Dashed line in A indicates position of transverse sections in B and C. Dashed line in B and C indicates embryonic midline. Adapted from (Horne-Badovinac et al., 2003)

lateral plate migrates ventro-laterally to the developing liver. This migration results in leftward displacement of the endoderm (Horne-Badovinac et al., 2003).

Characterisation of the *heart and soul* mutant, which carries a lesion in the aPKC gene, revealed defects in digestive tract looping and randomisation of organ chirality (Horne-Badovinac et al., 2001). Analysis of LPM morphology in these mutants revealed that the LPM fails to undergo polarisation, and exhibits defects in migration (Horne-Badovinac et al., 2003), resulting in defective displacement of the digestive tract. Altogether, this demonstrates important roles for LPM differentiation not only in organ specification, but in addition to direct the morphogenesis of the digestive tract.

1.2 - Pancreas development

Concomitant to the formation of the liver bud, development of neighbouring endodermal structures such as the pancreas is initiated. The adult pancreas is derived from two separate anlagen which fuse to form a single functioning organ. In zebrafish the two anlagen consist initially of the endocrine and exocrine pancreas (Field et al., 2003a). The endocrine pancreas will give rise to cells that secrete essential hormones for body homeostasis, while the exocrine pancreas will secrete digestive enzymes into the zebrafish intestine. This is in contrast to mouse, where the pancreas arises from two anlagen, which are both composed of endocrine and exocrine cells (reviewed in Kume, 2005).

Morphological events of endocrine pancreas formation in zebrafish

The first of the two anlagen to form is the endocrine islet, marked by the expression of the homeodomain gene *pdx1* (Biemar et al., 2001). Expression has been detected

from around 10 somites, at which time the endocrine cells are situated as two bilateral groups of loosely connected cells along the A-P axis of the endodermal rod at the level of the first and second somite (Fig 1.7). Shortly after, these cells begin to aggregate at the embryonic midline. Concomitantly, *insulin,* the first endocrine specific gene to be expressed in the endocrine pancreas, expression is initiated in a subset of cells. By 18 hpf, the endocrine cells have migrated to the midline, and the number of cells co-expressing *pdx1* and *insulin* has increased (Argenton et al., 1999; Biemar et al., 2001). Between 18 hpf and 24 hpf the endocrine cells begin to aggregate posteriorly, dorsal to the endodermal rod, forming a single endocrine islet (Biemar et al., 2001; Huang et al., 2001).

The endocrine islet is composed not only of *insulin*-expressing β-cells, but also *glucagon*-expressing α-cells, and *somatostatin*-expressing δ cells. These cell types differentiate sequentially: *somatostatin* expression is initiated at 16 somites, while *glucagon* expression is not observed until 21 hpf (Argenton et al., 1999; Biemar et al., 2001). At 24 hpf and beyond, the different cell types of the islet have a distinct organisation; the core of the islet is composed of β and δ cells, while the α cells are situated at the periphery of the islet.

Molecular control of endocrine islet formation

The initial specification and positioning of endocrine cells within the endoderm is regulated in part by Retinoic Acid (RA) signalling. Analysis of *neckless (nls)* mutant embryos, which have impaired function of Raldh2 (which is involved in RA synthesis), or embryos treated with RA receptor antagonists, reveals a failure in endocrine pancreas specification, suggesting a role for RA signalling in endocrine pancreas specification (Stafford and Prince, 2002). Conversely, treatment of wild

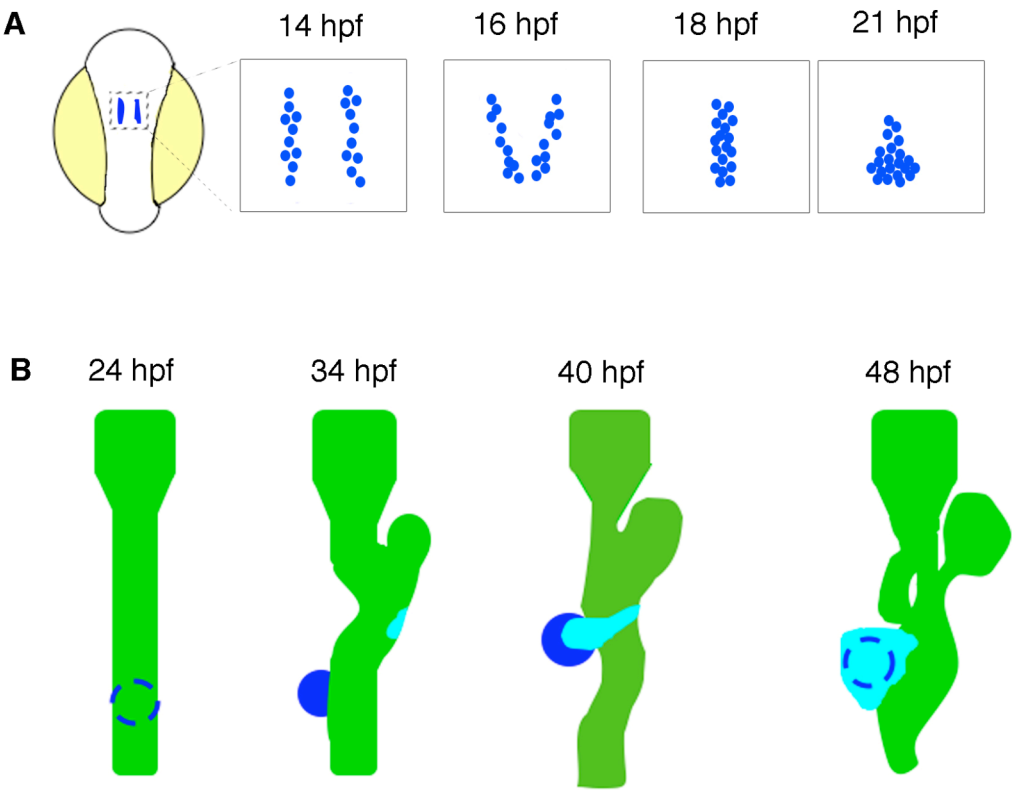


Figure 1.7 Pancreas formation in zebrafish

(A) The endocrine pancreas is initiated as bilateral groups of cells at around 14 hpf at the level of the first and second somites. These cells aggregate at the midline by 18 hpf, and migrate posteriorly to form a single pancreatic islet by 24 hpf. Dorsal views, anterior to top.

(B) The exocrine pancreas is initiated posterior to the liver at 34 hpf and subsequently projects to the right side of the midline by 40 hpf. At 48 hpf, the exocrine pancreas has engulfed the endocrine islet. Green, endoderm; blue, endocrine pancreas; cyan, exocrine pancreas. Ventral views, anterior to top.

type embryos with RA results in ectopic anterior clusters of cells, which arise as a consequence of aberrant anterior specification of endocrine cells (Stafford and Prince, 2002). Interestingly, high levels of exogenous RA treatment resulted in increased anterior shifting of all endodermal organs, including the endocrine pancreas, suggesting that RA regulates the anteroposterior patterning of the liver and pancreatic anlagen (Stafford and Prince, 2002). Importantly, exogenous RA treatment never resulted in posterior expansion of the endocrine pancreas, and recent studies have demonstrated that *cdx4* expression in the endoderm posterior to the pancreas may be sufficient to block RA signalling in this part of the embryo, directing localisation of the pancreas (Kinkel et al., 2008). Cell-autonomy studies demonstrated that RA signalling originates in the mesoderm adjacent to the foregut, and that receptors present in the endoderm transduce the RA signal (Stafford et al., 2006). This is similar to the proposed role for RA generated by Raldh1 and 2 in the mesoderm of mice that is required for endocrine pancreas induction (Kumar et al., 2003; Molotkov et al., 2005; Oström et al., 2008), highlighting a conserved role for RA signalling in endocrine specification in vertebrates. In addition it has been suggested that RA acts in concert with Bmp and Fgf signalling in endocrine pancreas specification, and these signals are mediated through regulation of *vhnf1* expression (Song et al., 2007).

In addition, initiation of endocrine pancreas specification in zebrafish requires *sonic hedgehog* (*shh*) activity. Analysis of endocrine pancreas formation in *slow muscle omitted (smu)* mutants, which lack all hedgehog signalling, revealed a dramatic reduction in the expression domain of *pdx1* and an absence of endocrine cell specification, as determined by a lack of Islet1, Insulin, or *nkx2.2* expression (Roy et al., 2001). Similar results were observed upon analysis of the *sonic you* (*syu*) mutant, which lacks *shh* signalling (diIorio et al., 2002). Furthermore, overexpression of *shh*

results in an expansion of *insulin* expression as the result of an increased number of β-cells, and these ectopic cells are often found in more anterior positions to the main endocrine islet at 24 hpf (diIorio et al., 2002; Roy et al., 2001) demonstrating that Hedgehog signalling, in particular *sonic hedgehog*, is required for endocrine pancreas formation.

The axial mesoderm is a potent source of *shh* signals, and *floating head* mutants that exhibit defects in formation of the axial mesoderm also display defective endocrine cell specification (Biemar et al., 2001; Chung and Stainier, 2008; Dong et al., 2008). Overexpression of *shh* in *flh* mutants can rescue defects in endocrine specification, confirming that *shh* activity in the axial mesoderm is required for endocrine specification (Chung and Stainier, 2008). Cell-autonomy analyses have demonstrated that at somitogenesis stages *shh* signalling from the mesoderm is transduced by lateral cells in the endodermal sheet, and that subsequent intra-endodermal interactions results in β-cell induction in more medial endodermal cells (Chung and Stainier, 2008).

Exocrine pancreas morphogenesis in zebrafish

In zebrafish the exocrine pancreas originates from a second independent primordium, initiated slightly posterior to the liver at around 34 hpf. This cell population expresses *ptf1a* - the earliest gene to be specifically expressed in the exocrine pancreas in zebrafish, and *pdx1* (Zecchin et al., 2004). Subsequently, the exocrine pancreas proliferates and projects to the right side of the embryonic midline, where it engulfs the endocrine pancreas from 40 hpf onwards (Argenton et al., 1999; Field et al., 2003a). The exocrine tissue then undergoes a period of growth, adopting a 'head and tail' conformation, where the 'head' surrounds the endocrine islet, and the 'tail'

extends posteriorly down the trunk of the embryo adjacent to the digestive tract. The combined exocrine and endocrine pancreas are joined to the adjacent foregut by the extrapancreatic duct and the common hepatopancreatic duct, from around 48 hpf onwards.

Exocrine pancreas specification in zebrafish

Similarly to liver specification, the LPM directly adjacent to the future exocrine pancreatic endoderm is the source of inductive factors required for exocrine pancreatic specification (Manfroid et al., 2007). *fgf10* and *fgf24* expressed in the pancreatic LPM, are necessary to induce exocrine tissue in the underlying endoderm (Manfroid et al., 2007). Interestingly, the same study also demonstrated that *fgf24* expression is first observed in the presumptive pancreatic endoderm, and is required to initiate *fgf10* and *fgf24* expression in the neighbouring LPM, as well as expression of target genes such as *meis3*, which is required for growth of the exocrine pancreas (Manfroid et al., 2007), highlighting the complex interactions between the endoderm and mesoderm required for pancreatic organogenesis.

Ptf1a, the earliest gene expressed in the zebrafish exocrine pancreas, has been implicated in exocrine differentiation, with *ptf1a* morphants lacking differentiated exocrine tissue (Lin et al., 2004). Interestingly, analysis of *ptf1a* expression in *ptf1a* morphants revealed that the exocrine pancreas programme is initiated, but arrests before 48 hpf, suggesting that *ptf1a* is required for maintenance or survival of the pancreatic population (Lin et al., 2004). Strikingly, recent studies of *ptf1a* hypomorphic embryos suggest that high levels of *ptf1a* expression are required to direct exocrine pancreas differentiation and to repress endocrine pancreas formation,

while low levels of *ptf1a* expression promote ectopic endocrine cells (Dong et al., 2008).

Thesis aims

The liver performs many essential functions in embryonic and adult vertebrates, including the secretion of bile into the digestive system, production of plasma proteins, and synthesis of clotting factors. Identification of novel factors regulating the specification, differentiation, morphogenesis and growth of the liver will further our understanding not only of the processes underlying hepatic development, but will be important for understanding the implications of misregulation of these events in disease and cancer.

The use of forward genetic screening in zebrafish as a tool to identify novel genes involved in developmental processes is well established (Amsterdam et al., 2004; Driever et al., 1996; Geisler et al., 2007; Golling et al., 2002; Haffter and Nusslein-Volhard, 1996). A forward genetic screen using N-ethyl-N-nitrosourea (ENU) mutagenesis, which introduces single base pair transitions transmissible in the germ line, was carried out to specifically identify genes involved in endodermal organogenesis in zebrafish. Mutagenesis was carried out in the *Tg(gutGFP)*^{s854} line expressing GFP in the endoderm under the control of the *foxA2* promoter (Field et al., 2003b) (Ng et al., 2005; Ober et al., 2006). Zebrafish embryos are transparent, therefore mutagenised transgenic lines expressing fluorescent proteins in the digestive system can be screened live at a variety of time points. This facilitates screening, allowing rapid identification of mutant lines with defects in endodermal organogenesis. Two mutant lines, *s436* and *4C1* were identified as part of this forward genetic screen. Initial analysis revealed that both lines exhibited liver

hypoplasia at 48 hpf, therefore promising to provide new insights into the molecular mechanisms underlying liver development.

In my thesis I focussed on elucidating the roles of *s436* and *4C1* in hepatic development in zebrafish. To address this, I characterised the morphological and molecular details of liver formation in these two mutant lines, within the context of the developing digestive system. To position both genes within the existing gene network, and to reveal the molecular mechanism underlying their function in hepatic development, positional cloning and cell autonomy studies were initiated.

The first part of my thesis focuses on the analysis of the *s436* mutant line, and the second on the *4C1* mutant line

Chapter 2

Materials and Methods

2.1 Fish Stocks

Adult zebrafish and embryos were raised according to standard laboratory conditions (Westerfield, 2000).

The following strains were used:

Zebrafish strain	Reference
hdac1^{s436}	Laboratory stock
hdac1^{hi1618}	(Golling et al., 2002)
Tg(gutGFP)^{s854}	(Field et al., 2003b)
Tg(lfabp:dsRed;elastaseA:GFP)	(Dong et al., 2007)
casanova^{ta56}	(Kikuchi et al., 2001)
4C1	Laboratory stock
SJD	Laboratory stock
WIK	Laboratory stock
Ekkwill	Laboratory stock
LonTuploff	Laboratory stock

2.2 Genetic mapping and positional cloning

A mapping strain was created by crossing a *hdac1*^{s436}*;Tg(gutGFP)*^{s854} female to a wild type SJD male. Bulk segregant analysis was performed on pools of 48 sibling and mutant embryos, using simple sequence length polymorphisms (SSLP; MWG Biotech; Nusslein-Volhard and Dahm, 2002) to map *s436* to linkage group 19. Subsequent fine mapping linkage analysis was performed on individual sibling and mutant embryos using SSLP primers spread throughout linkage group 19. Markers were identified using the Tuebingen (http://wwwmap.tuebingen.mpg.de), or MGH

(http://zebrafish.mgh.harvard.edu) websites. The following polymorphic markers were used to determine the genetic region encompassing the *s436* locus:

Marker	Position cM (Tuebingen map)	Primers
Z13773	48.4	F CCCTCTCCTCAACCATAGCA R CTGGCATGTAGCACCATACG
Z7235	48.9	F TGATTCAAAGCAGAGGCTGA R TTCAAGCACGGATTTGAAAA
Z15237	49	F AATCTGCACCAGCTGCAACG R GCACTGGCGTGAAGATCATA
Z22532	53.1	F GCTGGGAATAAAGGACACCA R CAGGTGCTGTTTTCAAGCAA
Z53477	54.2	F GCGATGTGGGTAAAAGGTGT R TACCTCCCGATCAGGACTTG
DKEY-108G22-76440*	57.8	F GAGGGTATCACCAGTGTCCTT R ATATGTCACGTCAGTGCTGGT
Z14487	59	F ACCTGGCATATGTACAGCCC R AGGATGCACCACATCCTTTC
Z6661	67.2	F CCTCCAGGATCCTGCAGAAA R TTCTCTGACTGCCCTCGG

*newly designed based on identified CA repeats

Complementation studies for *hdac1* were carried out by crossing heterozygous fish for the $hdac1^{hi1618}$ and the $hdac1^{s436}$ allele.

Two independent cDNA preparations were generated from mutant and wild type embryos using Superscript First Strand Synthesis System (Invitrogen). The Hdac1 open reading frame (ORF) was amplified by two overlapping PCR reactions, and six overlapping PCR reactions to ensure accurate sequence analysis. Two independent sequencing reactions were carried out per cDNA preparation.

2.3 Western blot analysis

Embryos were deyolked in chilled PBST (PBS, 0.1% Tween-20) and pools of 25 embryos for each genotype and stage frozen at -80°C. Embryos were homogenised in SDS-Gel loading buffer (100mM pH6.8, 4% SDS, 0.2% Bromophenol blue, 20% glycerol, 200mM β-mercaptoethanol) and heated at 98°C to extract proteins. Samples were spun at 4°C for 5 minutes, and the supernatant recovered. Protein samples were normalized using Coomassie Blue staining. Extracted proteins were run on a 12% SDS gel, and transferred to a PVDF membrane (BioRad). Membranes were blocked with 5% nonfat powdered milk in PBST and incubated with anti-hyperacetylated histone H4 antibody (1:1000, Upstate), or anti β-actin antibody (1:1000, Sigma) overnight at 4°C. Membranes were washed in blocking solution, and incubated with anti-rabbit HRP IgG antibody (1:5000, BioRad) for 30 minutes. After washing with blocking solution, blots were visualised using enhanced chemiluminescence (Amersham). Experiments were repeated with samples homogenised in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris pH8) and protein content determined using Bradford assay (Bradford, 1976) prior to gel loading. 0.44μg of protein was loaded per sample.

2.4 Immunohistochemistry

Embryos for immunostaining were fixed in 2% paraformaldehyde (PFA) overnight, and manually deyolked with forceps. Embryos were treated with DNAase (66mM Tris pH7.5, 5mM $MgCl_2$, 1mM β-mercaptoethanol, 50U/ml DNAase1) for 75 minutes, and following five brief washes with PBT (PBS, 0.1% Triton-X) were subsequently blocked in PBT with 10% Sheep Serum for 30-60 mins at 4°C. Primary antibodies were added overnight at 4°C or for 2 hours at room temperature (RT). Embryos were washed 3 x 5 min then 4 x 30 min in PBT at RT. Secondary antibodies

were added overnight at 4°C or for 2 hours at RT, followed by 3 x 5 min and 4 x 30 min PBT washes.

The following antibodies were used:

Antibody	Species	Dilution	Supplier/Reference
2F11	Mouse	1:1000	gift from Julian Lewis (Cancer UK; Dong et al., 2007)
Alcam	Mouse	1:15	Developmental Studies Hybridoma Bank
HNF4α	Mouse	1:100	Santa Cruz
Islet1/2	Mouse	1:15	Developmental Studies Hybridoma Bank
Phospho-histone H3	Rat	1:500	Abcam
aPKC	Rabbit	1:1000	Santa Cruz
GFP	Rabbit	1:500	Torrey Pines Biolabs
Laminin	Rabbit	1:100	Sigma
Prox1	Rabbit	1:1000	Chemicon

Fluorophore-conjugated secondary antibodies were used 1:200-1:500 (Jackson Laboratories, USA), fluorophore-conjugated phalloidin was used 1:50 (Sigma) and DAPI (Sigma) was used 1:4000. Embryos were mounted in 4% low-melting-point agarose/1% gelatine (Roche/Sigma). 130-140μm longitudinal sections and 80-100μm transverse sections were prepared using a Leica Vibratome and visualised using a Leica DMRXE SP1 or a Zeiss LSM5 Pascal Exciter confocal microscope. Images were processed using Volocity image analysis software (Improvision).

2.5 Quantification of endodermal cell number

Immunohistochemistry was carried out as described above. 90μm transverse sections were prepared, and optical stacks were taken using a Zeiss LSM5 Pascal Exciter confocal microscope throughout the organ-forming region. For each stack, cell

numbers were determined in three separate optical slices at fixed distance through the stack. The numbers of total endodermal nuclei, Prox1-positive hepatoblast nuclei, and Prox1-positive exocrine pancreatic nuclei were counted manually, using Volocity image analysis software for visualisation.

2.6 Whole mount in situ hybridisation

Embryos for in situ hybridisation were fixed in 4% PFA overnight and subsequently transferred into MeOH for storage at -20°C prior to experimentation. Embryos older than 24 hpf were treated with 0.2 mM 1-phenyl-2-thiourea (PTU) in egg water to inhibit melanin production. Embryos were rehydrated, and treated with Proteinase K:

Embryonic stage (hpf)	Protinease K treatment (min)	Proteinase K concentration (μg/ml)
< 24	0	N/A
24-26	8	10
28	10	10
30	20	10
30-40	30	10
48-56	45	10
>56	45	20

After brief PBST (PBS, 0.2% Tween-20) washes, embryos were re-fixed and placed in hybridisation buffer (50% formamide, 5X SSC, 0.1% Tween, 9mM citric acid pH6, 500μg/ml tRNA) for >1hr at 70°C. Probes were diluted 1:200 in hybridisation buffer and 200μl of probe solution was added per tube of embryos. Hybridisations were performed overnight at 70°C. All probes were generated per manufacturers instructions (Roche). Embryos were subsequently transferred into SSC buffer, then PBST, before being blocked for >1hr in blocking solution (2mg/ml BSA/PBST wih

5% Sheep Serum) at RT. Anti-DIG-AP antibody (Roche, 1:4000 in blocking solution) was added overnight at 4°C. Following antibody removal, embryos were washed 8 x 15min in 2mg/ml BSA/PBST, followed by 3 x 5min in staining buffer (100mM Tris pH9.5, 100mM NaCl, 50mM $MgCl_2$, 0.1% Tween-20). In situ hybridisations were developed using BCIP/NBT, fixed in 4% PFA for 2 hours at RT or overnight at 4°C, and dehydrated in MeOH. Embryos were cleared in BBA (Benzyl Benzoate:Benzyl Alcohol; 2:1).

Fluorescent in situ hybridisations were carried out using the TSA Plus Fluorescence palette system (Perkin-Elmer). Anti-digoxygenin-HRP (Roche) and anti-fluorescein-HRP (Perkin-Elmer) antibodies were used at 1:1000; tyramide-labelled substrate was used at 1:50.

The following probes were used;

Probe	Reference
ccne	(Yarden and Geiger, 1996)
cdkn1c	(Park et al., 2005)
ceruloplasmin	(Korzh et al., 2001)
claudin15	(Bagnat et al., 2007)
cyclinD1	(Yarden and Geiger, 1996)
dhand	(Angelo et al., 2000)
foxA1	(Odenthal and Nüsslein-Volhard, 1998)
foxA3	(Odenthal and Nüsslein-Volhard, 1998)
hdac1	(Pillai et al., 2004)
hdac3	Unpublished data, V. Cunliffe
hdac11	Unpublished data, V. Cunliffe
her5	(Müller et al., 1996)
hhex	(Ho et al., 1999)
insulin	(Milewski et al., 1998)

meis3	(Sagerström et al., 2001)
p27a	(Masai et al., 2005)
p27b	(Masai et al., 2005)
pcna	(Leung et al., 2005)
pes	(Allende et al., 1996)
ptf1a	(Lin et al., 2004)
prt/wnt2bb	(Ober et al., 2006)
sePb	(Thisse et al., 2003)
tbx5	(Begemann and Ingham, 2000)
trypsin	(Biemar et al., 2001)

2.7 Mosaic analysis

Wild type or *Tg(gutGFP)*^{s854} (donor) embryos were injected at the 1-cell stage with either 100-200 pg *casanova* sense RNA (generated using the mMESSAGE mMACHINE SP6 kit (Ambion) from the pCS2*cas* construct; Aoki et al., 2002) or 0.5mM *casanova* morpholino (5'-CAGGGAGCATCCGGTCGAGATACAT-3'; Dickmeis et al., 2001). In addition, donor embryos were co-injected with either rhodamine dextran or fluorescein dextran (Invitrogen) to trace cells post transplantation. Donor (wild type) or host (progeny of *hdac1* heterozygous crosses) were dechorionated manually and placed in agarose coated dishes. Cells were removed from wild type embryos at between 3-5 hpf and placed at the blastoderm margin of host embryos, where mesodermal and endodermal cells are located at this stage. Mosaic embryos were subsequently raised in Danieau buffer (0.8mM NaCl, 10mM KCl, 10mM $MgSO_4$, 25mM $NaHCO_3$, 50mM HEPES, pH 7.6) with Penicillin-Streptomycin (0.1%; Sigma) and fixed in 4% PFA.

2.8 TSA treatment

Trichostatin A (TSA, Invivogen) was resuspended in DMSO to 1 mg/ml, and diluted in egg water to concentrations between 200nM and 1200nM. Embryos were incubated at 28 °C in TSA (control embryos were treated with equal amounts of DMSO) in a shaking incubator. Final concentration of DMSO in experimental and control dishes was ≤2%.

2.9 Labelling for apoptosis

Embryos were fixed in 4% PFA and dehydrated in MeOH. After rehydration, embryos were treated with Proteinase K as described in Section 2.6, and immunolabelling was performed as previously described in Section 2.4. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (Tunel), using an In Situ Cell Death Detection Kit (Roche).

2.10 Isolation of zebrafish *vitamin D binding protein*

See Appendix 1

2.11 Morpholino injections

Morpholinos against *hdac3* (5' GAAGTACGCAGTTCGATTGGTCATG 3'), *hdac8* (5' AACACCACATAAACAGATACACAGC 3') and *hdac11* (5' TCTTTCTTTGACATTGTTGCCACAG 3') generated by GeneTools were a kind gift from V. Cunliffe. Morpholinos were diluted in 5mM Hepes and 0.5% Phenol Red; 5 or 10 ng were injected into wild type embryos or the progeny of *hdac1* heterozygote crosses between 1-cell and 4-cell stages.

Chapter 3

Hdac1 is required for endodermal organogenesis in zebrafish

3.1 The mutant line *s436* encodes a novel allele of *histone deacetylase 1*

The mutant line *s436* was identified as part of the forward genetic screen to identify mutants with defects in endodermal organogenesis (Ober et al., 2006). Initial analysis of *s436* mutant embryos using the *Tg(gutGFP)*^{s854} line revealed that at 48 hpf the liver is hypoplastic and the exocrine pancreas absent, in addition to morphogenetic defects within the digestive tract (Figure 3.1, E, F). *s436* mutants can be distinguished from wild type embryos from around 24 hpf onwards, by defects in body axis extension, eye and pigment formation and yolk absorption, as well as smaller fin buds and heart oedema (Fig. 3.1, A-D).

To understand the molecular nature of the *s436* phenotype, I set out to isolate the affected genetic locus in the *s436* mutant. Initially, a set of 192 simple sequence length polymorphism (SSLP) markers evenly distributed throughout the entire genome (Nüsslein-Volhard and Dahm, 2002) were used on pools of wild type and *s436* mutant embryonic genomic DNA to map the mutant locus to marker z6661 on linkage group 19. Single *s436* mutant embryos were analysed with polymorphic markers located north and south of Z6661 to determine the genomic region encompassing the lesion. Once this region was determined, recombinant analysis was used to further narrow it down, until analysis of 560 meiosis revealed one and nine recombination events for markers z15237 and z22532, respectively. This highlighted a region of approximately 4 cM (Fig. 3.2, A). The region these two markers encompasses contains approximately 40 genes, including *histone deacetylase 1* (*hdac1*). Several alleles of *hdac1* have been previously described, including the insertional mutant allele *hdac1*^{hi1618} (Golling et al., 2002), which exhibit similar

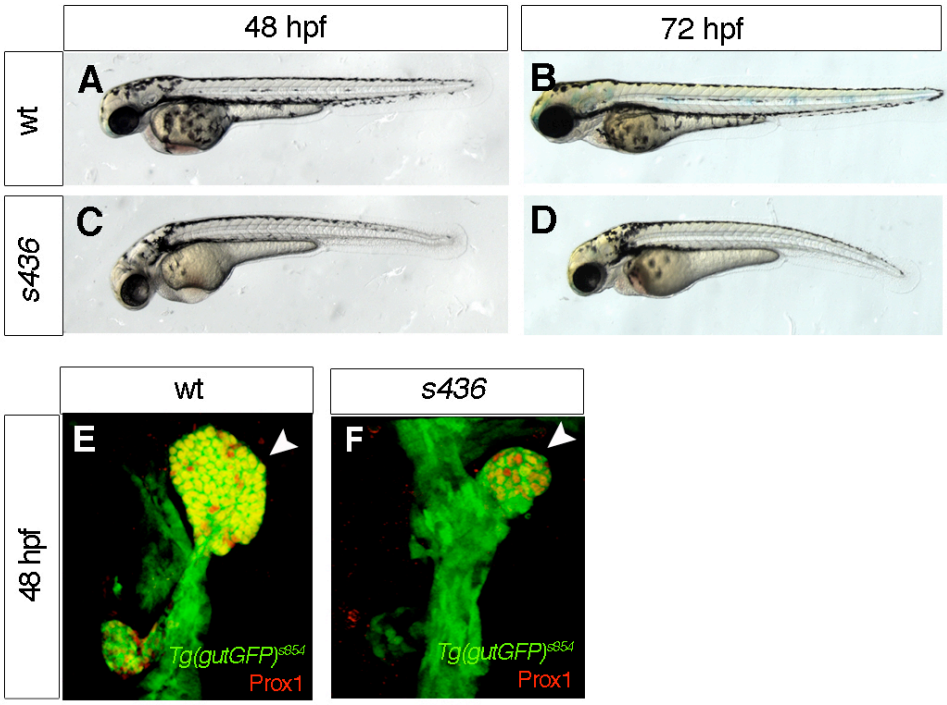


Figure 3.1 *s436* mutant embryos exhibit defects in endodermal organogenesis.

(A-D) Brightfield pictures of wild type (A,B), and *s436* mutant (C,D) embryos at 48 and 72 hpf (E-F) Confocal projections of ventral views of the organ-forming region of wild type (E) and *s436* mutant (F) embryos using the *Tg(gutGFP)*^{s854} line (green) highlighting the endoderm, in conjunction with α-Prox1 (red) labelling the liver and exocrine pancreas. Arrowhead, liver.

external body phenotypes to those observed in *s436* mutants, such as heart, fin, pigmentation and body shape defects (Fig. 3.1; Golling et al., 2002; Nambiar et al., 2007; Stadler et al., 2005; Yamaguchi et al., 2005). To determine whether *s436* may encode a novel allele of *hdac1,* I performed a complementation test crossing heterozygous *s436* fish with those heterozygous for the *hdac1*^{hi1618} allele. *s436* failed to complement the *hdac1*^{hi1618} allele, suggesting that *s436* represents a novel allele of *hdac1*. To isolate the lesion in *s436* mutants, the *hdac1* gene product was sequenced from *Tg(gutGFP)*^{s854} and *s436* mutant embryos, and a T to A point mutation was identified at position 800 (Fig 3.2, B, C), resulting in the replacement of a conserved leucine with a glutamine at amino acid 267 (Fig. 3.2, D). Hdac1 is highly conserved between vertebrates, with 96% similarity between the catalytic domains of zebrafish and human (aa22-322). Leu267, corresponding to Leu265 in humans, is located in the binding pocket of the catalytic active site of Hdac1 (Fig. 3.2. D; Finnin et al., 1999). The position of the point mutation in *hdac1*^{s436} within the catalytic domain suggests that catalytic deacetylase function may be impaired.

To determine changes of deacetylase activity in *s436* mutants, I compared the levels of histone acetylation in wild type and mutant embryos. Western blot analysis of wild type and *s436* mutant protein extracts using an anti-hyperacetylated histone H4 antibody revealed increased levels of histone acetylation in *s436* embryos when compared to levels in wild type embryos at 28 and 48 hpf (Fig. 3.2, E). Additionally, levels of histone H4-acetylation in *s436* mutants were similar to levels observed in *hdac1*^{hi1618} mutants at these stages. The *hdac1*^{hi1618} insertional allele is thought to be a null allele, therefore the similar increases in levels of histone acetylation in both *hdac1* mutant alleles supports the possibility that impairment of catalytic function in *hdac1*^{s436} mutant embryos results in a functionally inactive protein. Moreover, the

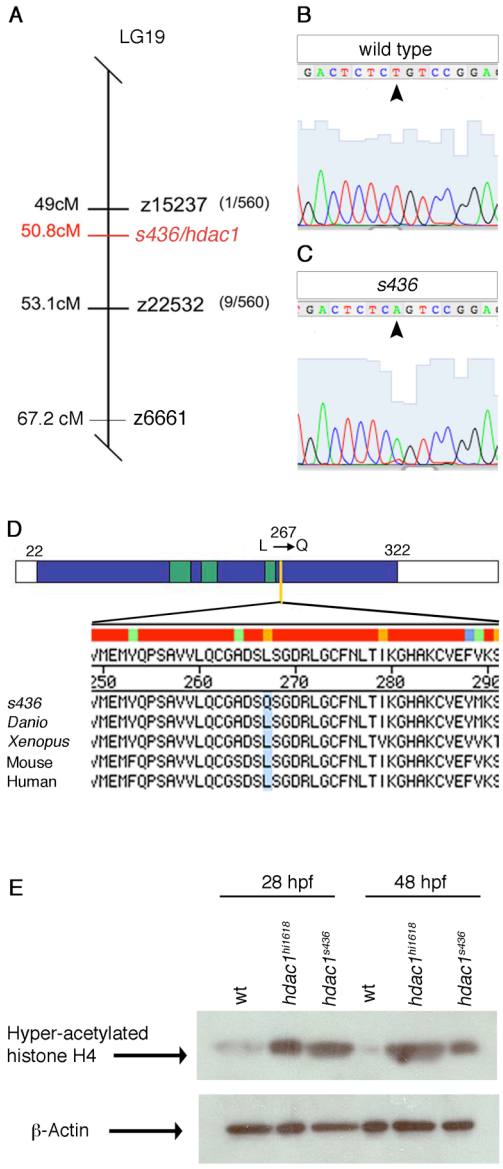


Figure 3.2 *s436* encodes a novel allele of *histone deacetylase 1.*

(A) Schematic representation of the position of the *s436* lesion on linkage group 19, with north and south microsatellite markers. The *hdac1* gene lies within this region. (B-C) Sequence analysis of the *histone deacetylase1* gene product in wild type (B) and *s436* mutant (C) embryos reveals a T-A base pair transition (arrowhead) at position 800. (D) Schematic representation of the predicted domain structure of the zebrafish Hdac1 protein, with catalytic region depicted in blue, Hdac superfamily regions in green, and the position of the *s436* lesion in yellow. (E) Western blot analysis of levels of hyper-acetylated histone H4 protein in wild type, *hdac1*^{s436} and *hdac1*^{hi1618} mutants, with β-Actin as a loading control.

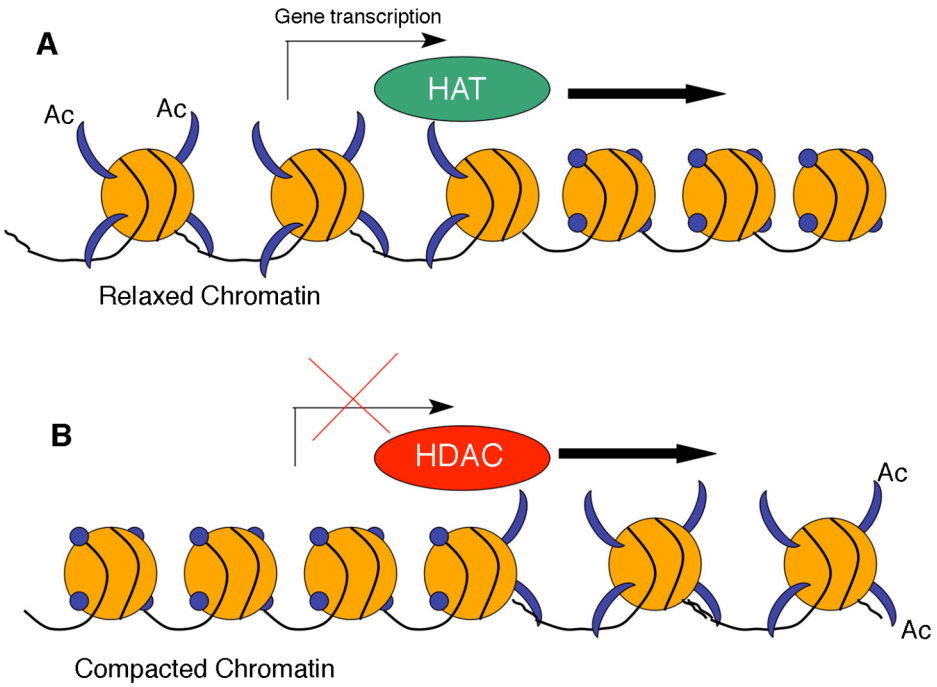


Figure 3.3 Transcription is regulated by histone acetylation

(A) Histone acetylation complexes (HAT) move along compacted chromatin adding acetyl groups to lysine tails of Histone H4. This acetylation results in a more relaxed chromatin conformation, allowing access of transcriptional regulators to their DNA binding sites. (B) Conversely, Histone deacetylation complexes (HDAC) move along open chromatin removing the acetyl groups from Histone H4. This results in a more compacted chromatin conformation, preventing access of transcriptional regulators to bind to the DNA. Modified after de Ruijter et al., 2003.

expression of four different genes, expressed in the forming hepatoblasts, differentiating liver, early exocrine pancreas and pancreatic β-cells, was compared between *hdac1*^{s436} mutants and mutants for the insertional allele, *hdac1*^{hi1618} (progeny of F1-carriers in the *Tg(gutGFP)*^{s854} background). This analysis revealed qualitatively highly similar phenotypes of differing penetrance for the examined organs (Appendix 2). Notably, these differences in penetrance may not be due to differences in impairment of catalytic function between the two *hdac1* alleles, as phenotypic analysis of the *hdac1*^{hi1618} mutation in different genetic backgrounds has revealed variable phenotypic severity (Pillai et al., 2004). This suggests that differences in phenotypic penetrance between the two *hdac1* alleles may be the result of differences in genetic background, not differences in loss of catalytic activity. Taken together, these data confirm that *s436* encodes a loss-of-function allele of *hdac1*, likely a null allele, hereafter referred to as *hdac1*.

Chromatin modifications, such as methylation or acetylation are central to regulating gene expression (Jenuwein and Allis, 2001; Kouzarides, 2007). DNA methylation represents a stable and heritable mechanism for epigenetic silencing of transcription (Goll and Bestor, 2005). In contrast, histone acetylation mediated by histone acetyltransferases prevents chromatin condensation, thus allowing transcriptional activation. Conversely, removal of acetyl groups leads to chromatin compaction resulting in transcriptional repression, and this deacetylation is mediated by Histone deacetylases (Fig 3.3). The temporally and spatially coordinated regulation of gene expression is critical during the development of a multi-organ structure such as the digestive system requires. Neighbouring groups of cells, which initially share a common gene expression programme, will adopt different fates by subsequently expressing different sets of genes. This is realised by active regulation of initiation

and termination of transcription, which in turn depends on the presence of specific activating and repressing transcription factors, and their ability to access regulatory gene elements.

In order to determine *hdac1* expression during endodermal organogenesis, I performed in situ hybridisation on wild type and *hdac1* mutant embryos. *Hdac1* is expressed ubiquitously throughout the embryo from the one cell stage, suggesting maternal contribution (Cunliffe, 2004; Yamaguchi et al., 2005). At 24 hpf, *hdac1* is still expressed uniformly throughout the embryo (Figure 3.4, A). However, over the course of the next 48 hours expression becomes restricted to more anterior tissues (Fig. 3.4, B-D). At this time, expression can be observed in the area of the liver and digestive tract (arrowhead and dotted line, D). Analysis of *hdac1* expression in *hdac1* mutants revealed reduced expression of *hdac1* in the fin buds (Fig. 3.4, E-H), suggesting that similar to reports from analysis of the murine Hdac1 promoter (Schuettengruber et al., 2003), Hdac1 is involved in auto-regulation of its expression in this tissue.

Liver formation arises from interactions between the endoderm and neighbouring mesoderm, therefore I wished to determine whether *hdac1* is predominantly expressed in endodermal or mesodermal tissues during organogenesis. Analysis of sectioned *hdac1* in situ hybridisations were inconclusive, and antibodies against zebrafish Hdac1 were not available. To determine the tissues in which *hdac1* is expressed during endodermal organogenesis, the use of fluorescent in situ hybridisation in conjunction with confocal microscopy may prove more successful, facilitating the identification of mesodermal and endodermal tissues.

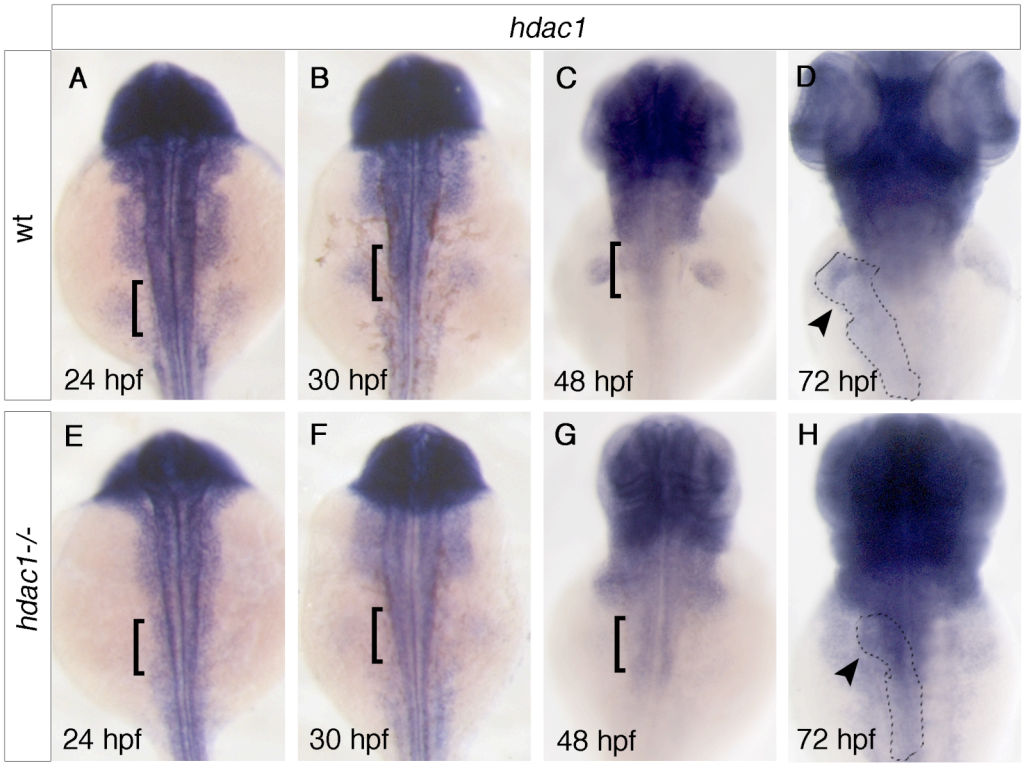


Figure 3.4 *hdac1* expression during endodermal organogenesis.

(A-H) In situ hybridisation for *hdac1* gene expression in wt (A-D) and *hdac1* mutant embryos (E-H). Dorsal views, anterior to top. Brackets indicate organ-forming region, arrowheads denote fin buds, dotted line outlines liver and intestine.

3.2 Hdac1 plays multiple roles in endodermal organogenesis

hdac1 mutant embryos exhibit a hypoplastic liver at 48 hpf (Fig. 3.1, E, F). To determine when the onset of defects in liver organogenesis in the context of the developing digestive system are first detected in *hdac1* mutant embryos, early stages of endodermal organogenesis were analysed using the *Tg(gutGFP)*^{s854} line, expressing GFP throughout the endoderm (Field et al., 2003b), in conjunction with antibodies against Prox1 to highlight specified hepatoblasts and exocrine pancreatic cells, and Islet 1/2, expressed in the endocrine pancreas and at low levels in the neighbouring mesoderm. In wild type embryos at 24 hpf, Prox1 is expressed by newly specified hepatoblasts throughout the liver-forming region of the ventral endoderm (Fig. 3.5, B; Ober et al., 2003). Prox1 expression is maintained in the budding liver, visible at 30 hpf (Fig. 3.5, C), and subsequently in the differentiating liver at 48 hpf (Fig. 3.5, E). In contrast, during hepatic development in *hdac1* mutants, Prox1 expression in the hepatic endoderm is absent at 24 hpf, and is not initiated until 28 hpf (Fig. 3.5, F, G). At this stage, its pattern of expression is similar to wild type embryos, however the number of cells expressing Prox1, as well as its expression levels, are strongly reduced. Moreover, looping of the digestive tract, which occurs in wild type embryos at 30 hpf, fails to be initiated at this stage in *hdac1* mutant embryo (compare Fig. 3.5 C, G). Prox1 positive hepatoblasts aggregate to form a medial liver bud by 40 hpf in *hdac1* mutants, in contrast to wild type embryos where the liver is positioned on the left side of the midline (Fig. 3.5, D, H). Subsequently, the liver in *hdac1* mutants becomes located asymmetrically on the left side at 48 hpf, however the liver bud is reduced in size when compared to wild type embryos (Fig. 3.5, I). This reduction in liver size at 48 hpf is accompanied by an apparent widening of the non-hepatic

foregut endoderm (Fig. 3.5, E, I). Digestive tract looping appears to have been initiated by 48 hpf in *hdac1* mutant embryos (Fig. 3.5, I), however the displacement of the intestine appears incomplete when compared to wild type embryos, highlighting a role for Hdac1 in morphogenesis of the digestive tract.

Concomitant with the described defect in liver formation, in *hdac1* mutant embryos the exocrine pancreas is absent at 48 hpf (Fig. 3.1, F). In wild type embryos the Prox1-positive exocrine pancreas is initiated at around 34 hpf in the ventral endoderm slightly posterior to the forming liver (Fig. 3.5, D). The exocrine pancreas subsequently undergoes morphogenesis and outgrowth, and by 48 hpf projects asymmetrically to the right side of the embryo where it engulfs the endocrine islet to form a single pancreatic anlage. In *hdac1* mutants exocrine pancreas formation is not initiated at 34 hpf, and is not detectable at 48 hpf (Fig. 3.5, H, I). To determine whether the defects in pancreas formation in *hdac1* mutants are specific to the exocrine pancreas, I analysed endocrine pancreas formation. The endocrine pancreas is a cluster of cells located at the posterior end of the organ-forming region at 24 hpf, and is Islet 1/2 positive (Fig, 3.5, B-E). In *hdac1* mutants, the endocrine islet forms, however ectopic anterior clusters of Islet 1/2-positive cells are detected at all stages examined (Fig. 3.5, F-I).

Taken together, these results suggest that *hdac1* is required for specification and morphogenesis of the liver, and additionally for exocrine pancreas specification and endocrine islet morphogenesis.

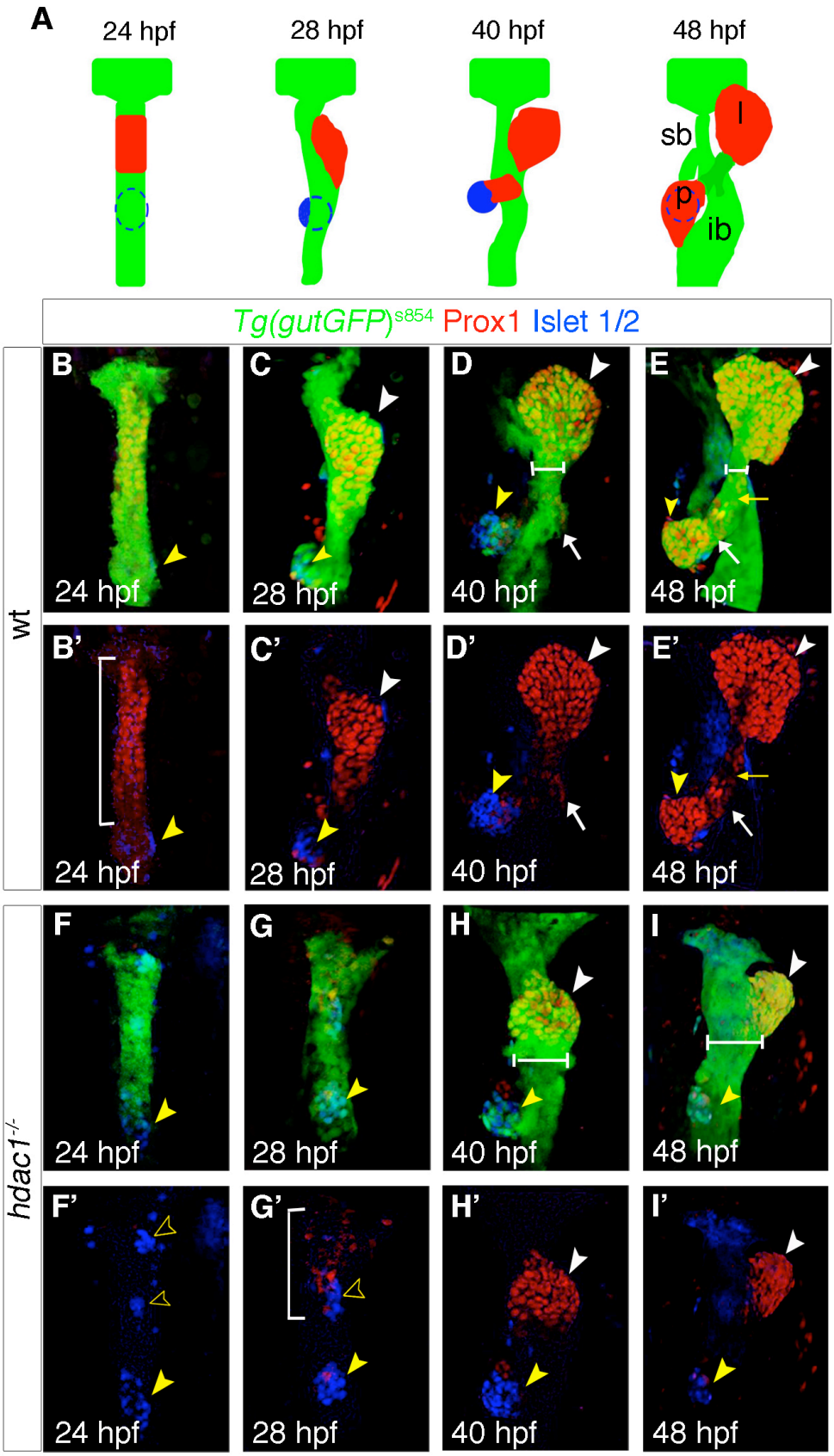

A
24 hpf
28 hpf
40 hpf
48 hpf
l
sb
p
ib
Tg(gutGFP)s854 Prox1 Islet 1/2
wt
hdac1-/-
B
C
D
E
B'
C'
D'
E'
F
G
H
I
F'
G'
H'
I'
24 hpf
28 hpf
40 hpf
48 hpf

Figure 3.5 Specific requirements for Hdac1 in endodermal organogenesis.

(A) Schematic depicting stages of endodermal organogenesis in wild type embryos. Endoderm in green, Prox1-positive liver and exocrine pancreas in red, and endocrine pancreas in blue, l, liver; p, pancreas; sb, swim bladder; and ib, intestinal bulb. (B-I') Time course analysis comparing endodermal organogenesis of wild type siblings and *hdac1* mutants, using the *Tg(gutGFP)*^{s854} transgene expressing GFP throughout the endoderm (green), stained for Prox1 (red) and Islet1/2 (blue) expression. Projection of confocal stacks showing ventral views of the organ-forming endoderm, anterior to top. (B-E') Wild type siblings. (F-I'), *hdac1* mutants, (B'-I' showing Prox1/Islet1/2 channels for clarity). (B, B') At 24 hpf Prox1-expressing hepatoblasts are found throughout the organ-forming region (brackets) of the endodermal rod. The endocrine pancreatic islet, stained with Islet1/2, is situated posteriorly in the organ-forming region (yellow arrowhead). (C, C') By 28 hpf the Prox1-positive hepatoblasts aggregate on the left side of the endodermal rod (white arrowhead) forming the liver bud. (D, D') The exocrine pancreas expresses Prox1 at 40 hpf (arrow) and 48 hpf; the liver and exocrine pancreas are connected to the adjacent digestive tract by the extrahepatopancreatic duct (yellow arrow, E, E'). (F, F') *hdac1* mutants lack Prox1 expression at 24 hpf. Additionally to the main endocrine islet, anterior groups of endocrine cells have formed (empty arrowheads). By 28 hpf, Prox1 expression is initiated in hepatoblasts (bracket, G'), which subsequently aggregate to form a medial liver bud by 40 hpf (white arrowhead, H, H'). By 48 hpf, in *hdac1* mutants, the liver bud is located asymmetrically on the left side of the digestive tract (white arrowhead, I, I'). The exocrine pancreas and swim bladder fail to form at this time. Additionally, the width of the digestive tract is greater in *hdac1* mutants than in wild type embryos once liver bud outgrowth has been initiated (compare horizontal bars D, E with H, I).

3.3 Hdac1 is required for timely liver specification

Analysis of liver development in *hdac1* mutants using the *Tg(gutGFP)*^{s854} line in conjunction with α-Prox1 staining suggested a defect in timely specification of nascent hepatoblasts. To determine whether this delay is specific to Prox1 expression alone, or indicative of a general defect in hepatoblast specification, expression of the homeobox transcription factor *hhex* was examined. *hhex* is expressed in newly formed hepatoblasts from around 22 hpf onwards (Fig. 3.6, A-C; Liao et al., 2000; Ober et al., 2003; Ober et al., 2006). Consistent with the defects observed in Prox1 staining, *hhex* expression is absent in the liver-forming region of *hdac1* mutant embryos at 24 hpf, and reduced at 30 and 48 hpf (Fig. 3.6, D-F).

Hepatoblast specification in mouse, chick and zebrafish occurs in part through signals that originate from the neighbouring mesoderm (Jung et al., 1999; Ober et al., 2006; Zhang et al., 2004). As the observed defect in hepatoblast specification could be the result of a reduction of mesodermally-derived specification factors, I examined *prt/wnt2bb* expression. In wild type embryos, *prt/wnt2bb* is expressed bilaterally in the LPM adjacent to the presumptive hepatic endoderm from around 18 hpf onwards, and has been shown to promote liver specification in zebrafish (Fig. 3.6, G-H; Ober et al., 2006). I observed reduced *prt/wnt2bb* expression in the LPM of *hdac1* mutants at both 26 hpf and 28 hpf (Fig. 3.6, I, J) likely accounting for the defect in the onset of hepatoblast specification.

3.4 Liver differentiation and extrahepatic duct formation require Hdac1 function

In wild type embryos hepatic specification is followed by differentiation of hepatoblasts into mature hepatocytes and cholangiocytes, a process which begins at

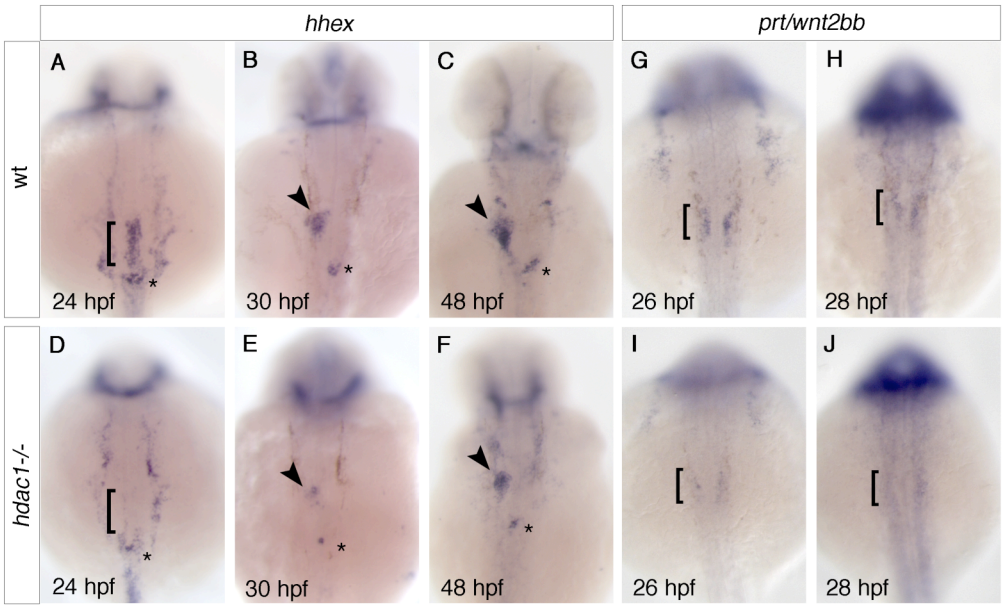


Figure 3.6 Hdac1 is required for timely liver specification.

In situ hybridisation analyses of *hhex* and *prt/wnt2bb* expression in wild type and *hdac1* mutants, dorsal views, anterior to top. Wild type embryos express *hhex* in hepatoblasts at 24 hpf (bracket, A), 30 hpf and 48 hpf (arrowheads, B, C). Additionally, *hhex* is expressed in pancreatic tissue (asterisk). *hdac1* mutants lack hepatic *hhex* at 24 hpf (bracket, D), although retain pancreatic expression (asterisk). Reduced hepatic *hhex* expression is observed in *hdac1* mutants at 30 hpf and 48 hpf (arrowhead, E, F). Wild type embryos express *prt/wnt2bb* in the LPM at 26 and 28 hpf (brackets, G, H), whereas *hdac1* mutants show reduced *prt/wnt2bb* expression (brackets, I, J).

around 34 hpf and is accompanied by the onset of expression of hepatic differentiation markers (Korzh et al., 2001; Thisse et al., 2003). Hepatoblast specification occurs in *hdac1* mutants, albeit with a delay. Therefore, to assess whether Hdac1 is also required for timely differentiation of the liver I examined the expression of a number of genes expressed in differentiating hepatoblasts. *ceruloplasmin (cp)* and *selenoprotein Pb (sePb)* are plasma proteins expressed in differentiating hepatoblasts from 34 hpf onwards (Fig 3.7, A-E; Korzh et al., 2001; Kryukov and Gladyshev, 2000). *hdac1* mutants fail to express *cp* and *sePb* at 48 hpf, indicating a failure in hepatic differentiation (Fig. 3.7, F, I). Notably, at 3 and 4 dpf *cp* expression can be detected in 30% (n=16) and 50% (n=18), and at 4 dpf *sePb* is detected in 45% (n=11) of *hdac1* mutant embryos, implying that hepatic differentiation proceeds in a subset of embryos (Fig. 3.7, G, H). Importantly, the domain of expression of these genes is reduced in size at both these stages suggesting an additional role for Hdac1 in growth of the liver during later stages of embryogenesis.

Hepatic differentiation was examined further by using a transgenic reporter line expressing dsRed under the control of the *liver fatty acid binding protein (lfabp)* promoter in the liver from 3 dpf onwards (Fig. 3.7, P, Q; Dong et al., 2007). While at 3 dpf *hdac1* mutant embryos fail to express dsRed (n=18), expression is detected in a subset of embryos at 4 dpf, and by 5 dpf in 45% of embryos (n=13, Fig. 3.7 S,T), similar to *cp* and *sePb* expression in *hdac1* mutants at 4dpf. Furthermore, *vitDbp*, a marker of functional hepatic differentiation, which is expressed in the liver of wild type embryos from around 60 hpf onwards, (Fig 3.7, K, L). is absent in *hdac1* mutants at both 72 hpf and 96 hpf (Fig. 3.7, M, N, n=12, n=6), confirming that hepatic differentiation is severely affected in these mutants, and suggesting that while

intermediate stages of hepatic differentiation occur, terminal differentiation is defective.

Liver function requires not only differentiation of hepatocytes, but in addition the formation of the intrahepatic biliary system and the extrahepatic duct to connect the liver to the adjacent digestive tract. The formation of the intrahepatic ducts from bipotent hepatoblasts is tightly connected to hepatic differentiation (Deutsch et al., 2001; Dong et al., 2007; Hunter et al., 2007). Due to the defects observed in differentiation of hepatoblasts into mature hepatocytes in *hdac1* mutants, in addition to the absence of a morphologically distinct extrahepatopancreatic duct at 48 hpf as observed using the *Tg(gutGFP)*^{s854} line (Fig. 3.5, E, I), I analysed formation of the ductal system by investigating 2F11 expression between 40 hpf and 4 dpf. Initially, 2F11, which recognises a currently unidentified protein, is expressed throughout the forming liver, both pancreatic anlagen and the presumptive ductal tissue, and over time becomes downregulated in differentiating hepatocytes and restricted to the forming ducts (Fig. 3.7, O-Q; Dong et al., 2007). Similar to the onset of hepatic gene expression, I observe reduced 2F11 expression in *hdac1* mutants when compared to their siblings at 40 hpf (n=8, data not shown). However, at 48 hpf a strong posterior expansion of the 2F11 expression domain is observed in *hdac1* mutants (Fig 3.7, R), which appears to have failed to undergo morphogenesis when compared to wild type siblings. From 48 hpf onwards in wild type embryos, 2F11 expression is heightened in the extrahepatopancreatic duct (Fig. 3.7 O-Q). Similarly, in *hdac1* mutants, I observed heightened 2F11 expression in the extrahepatopancreatic ductal tissue at 3 and 4 dpf (86%, n=6, 100%, n=9, respectively, Fig. 3.7 S, T), however cellular and ductal morphology is altered, and lumen formation appears impaired. In addition,

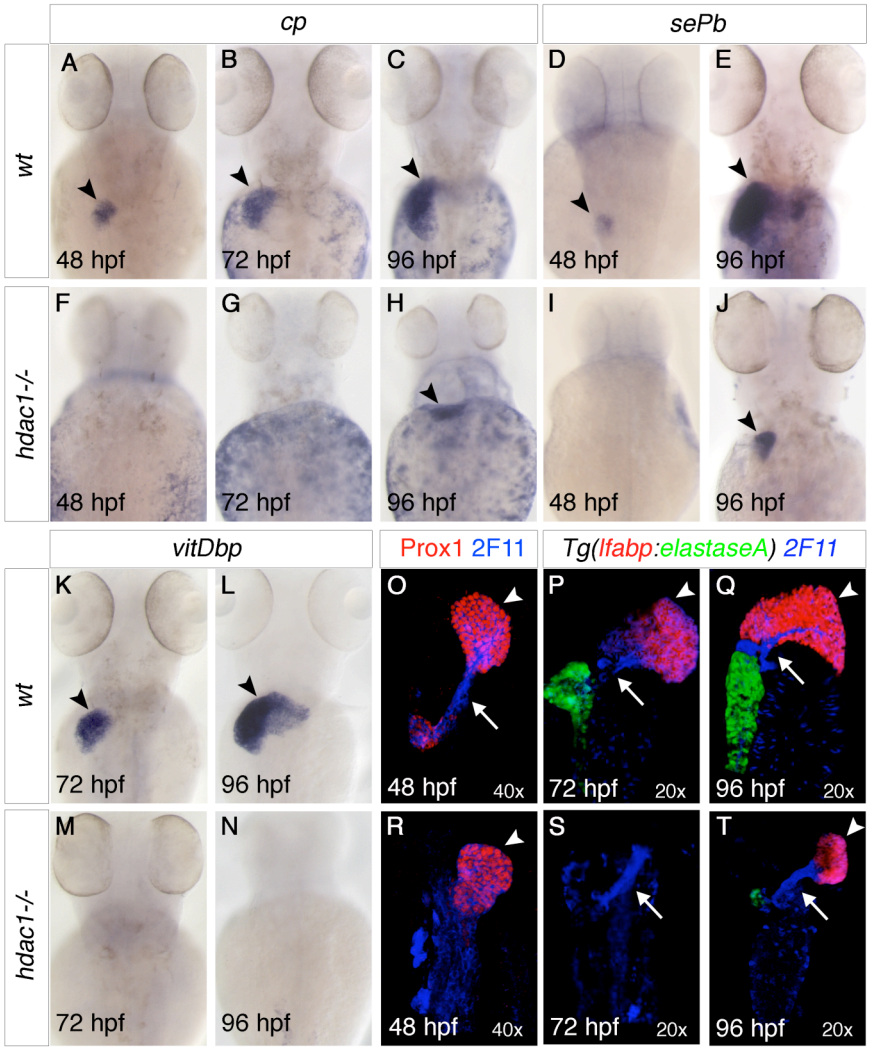


Figure 3.7 Hdac1 is required for timely liver differentiation.

(A-N) In situ hybridisation analyses of *cp, sePb* and *vitDbp* expression in wild type and *hdac1* mutants, dorsal views, anterior to top. Wild type embryos express *cp* and *sePb* in the liver from around 34 hpf onwards (arrowheads, A-E). *hdac1* mutants fail to express *cp* at 48 or 72 hpf (F, G), or *sePb* at 48 hpf (I). A subset of *hdac1* mutants express *cp* and *sePb* at 96 hpf (arrowhead, H, J). Wild type embryos express *vitDbp* in the liver from around 60 hpf onwards (arrowhead, K,L), however, *hdac1* mutants fail to express *vitDbp* at 72 hpf and 96 hpf(M, N). (O-T) Ventral projections of confocal stacks. Analysis of *Tg(lfabp:dsRed;elastaseA:GFP)* embryos (O-T) stained with α-2F11 reveals that in wild type embryos 2F11 expression is heightened in the hepatopancreatic ductal system at 48 hpf, 3 and 4 dpf (arrow, O-Q). In addition, wild type embryos express dsRed in the liver and GFP in the pancreas at 3 and 4 dpf (P, Q). In contrast, in *hdac1* mutants, 2F11 only becomes heightened in the dysmorphic extrahepatopancreatic ducts at 3 dpf (arrow, R-T). Additionally, only a subset of *hdac1* mutants express dsRed and GFP at 4 dpf (arrowhead, T).

refinement of the intrahepatic ductal system fails to occur in *hdac1* mutant embryos at these stages,. Notably, 2F11 expression in the extrahepatic duct in *hdac1* mutants is not dependent on hepatic differentiation as indicated by absence of dsRed expression under the control of *lfabp* (Fig. 3.7, S)

Altogether, these data suggest that *hdac1* is crucial for timely liver specification and differentiation. Similarly, *hdac1* is required for the elaboration of the intrahepatic ductal system and formation of the adjacent extrahepatopancreatic ducts. Strikingly, liver specification occurs, with a set back of approximately 6 hours while the subsequent lag of at least 50 hours in hepatic differentiation in *hdac1* mutants appears to be more severe and functional differentiation of the liver and intrahepatic ducts is defective.

3.5 Histone deacetylases are required during late somitogenesis for liver formation

Hdac1 is widely expressed during embryonic development and is required for multiple processes of endodermal organogenesis. This raises the question as to whether the observed defects are specific to endodermal organogenesis or are secondary to defects in early endoderm development. Expression of early endodermal genes *sox17* and *her5* (Alexander and Stainier, 1999; Dickmeis et al., 2001) was examined at epiboly stages and no differences in expression were observed between *hdac1* mutants and wild type embryos (data not shown). Similarly, *foxA1,* which is expressed pan-endodermally at 24 hpf (Odenthal and Nüsslein-Volhard, 1998), was expressed as in wild type in *hdac1* mutant embryos at 24 hpf (data not shown). These data indicate that early endoderm formation and endodermal rod morphogenesis occurs wild type-like in *hdac1* mutants.

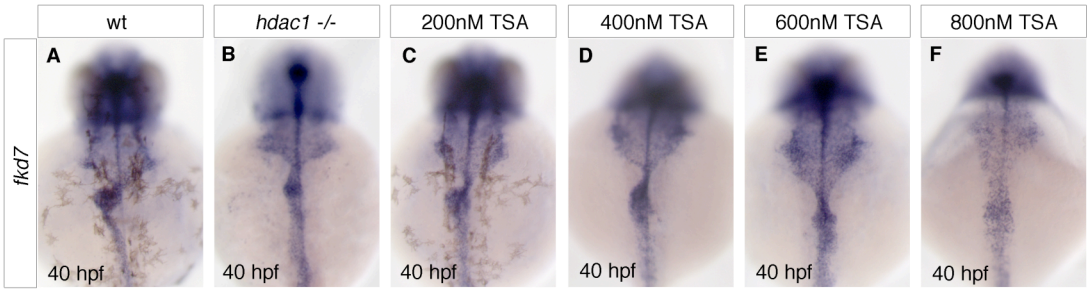


Figure 3.8 Treatment of wild type embryos with 600nM TSA recapitulates the *hdac1* mutant phenotype

(A-F) TSA was applied to wild type embryos at 14 hpf at concentrations ranging between 200nM and 1200nM, and digestive tract morphogenesis was assessed at 40 hpf by *foxA1* expression. 600nM TSA produced the closest phenocopy of the *hdac1*^{s436} phenotype when assessed by *foxA1* expression (compare panel E and B with A).

To determine the time of Hdac requirement during liver development, I used timed treatments of wild type embryos with Trichostatin A (TSA), which inhibits class I and class II Hdacs (Yoshida et al., 1995). Digestive tract morphogenesis and liver specification and differentiation were subsequently examined by *foxA1*, *hhex, prt/wnt2bb*, *cp* and *vitDbp* expression, respectively.

Wild type embryos were treated with TSA concentrations ranging between 200nM and 1200nM as previously described (Yamaguchi et al., 2005). Treatment with 600nM TSA closely phenocopied *hdac1*^{s436} defects when assessed by *foxA1* expression at 40 hpf and this concentration was used in subsequent experiments (Fig. 3.8, B, E). To elucidate Hdac function in liver specification, TSA was applied at various time points, and *hhex* expression was examined at 24 and 48 hpf. Application of TSA at 14 hpf resulted in an absence of hepatic *hhex* expression at both 24 and 48 hpf (Fig. 3.9, B, E). However, treatment at 18 hpf resulted in wild type expression of hepatic *hhex* at 24 hpf and a reduced domain of hepatic *hhex* expression at 48 hpf (Fig. 3.9, compare A, C, D, F). My data implicated *hdac1* in regulation of the mesodermally derived *prt/wnt2bb* expression, therefore I determined the time for Hdac requirement in directing *prt/wnt2bb* expression. Similarly to above, TSA application at 14 and 16 hpf resulted in an absence of mesodermal *prt/wnt2bb* expression at 26 hpf (Fig. 3.9, H and data not shown), while *prt/wnt2bb* expression was present in embryos treated at 18 hpf (Fig. 3.9, I). Altogether, these data support a requirement for Hdacs between 14 hpf and 18 hpf in hepatic specification.

To determine the temporal requirement for Hdacs in hepatic differentiation, TSA treated embryos were scored for *cp* expression at 48 hpf. Embryos treated at 14 hpf showed no *cp* expression at 48 hpf (Fig. 3.10, B), while the majority of embryos

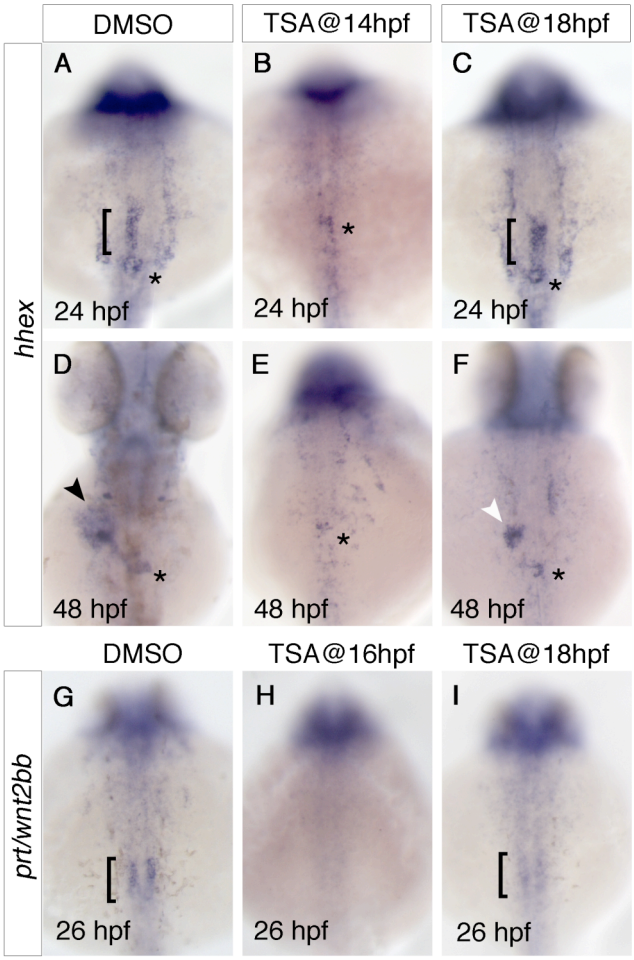


Figure 3.9 Histone deacetylase function is required during late somitogenesis for liver specification

TSA was applied at various time points and liver specification was examined by in situ hybridisation for *hhex,* and *prt/wnt2bb* expression. (A-F) Hepatoblast specification in TSA treated embryos was assessed by *hhex* expression. Application of TSA at 14 hpf resulted in an absence of hepatic *hhex* expression at both 24 and 48 hpf (B, E). However, treatment at 18 hpf resulted in wild type expression of hepatic *hhex* at 24 hpf (bracket, C), although strongly reduced at 48 hpf (arrowhead, F). (G-I) *prt/wnt2bb* expression was assessed in TSA treated embryos. TSA treatment at 16 hpf resulted in an absence of *prt/wnt2bb* expression in the LPM abutting the organ-forming endoderm (H), however, application of TSA at 18 hpf resulted in wild type-like expression of *prt/wnt2bb* (bracket, I). DMSO was added as a control in all cases (A, D, G); asterisk indicates position of the pancreas, anterior to top.

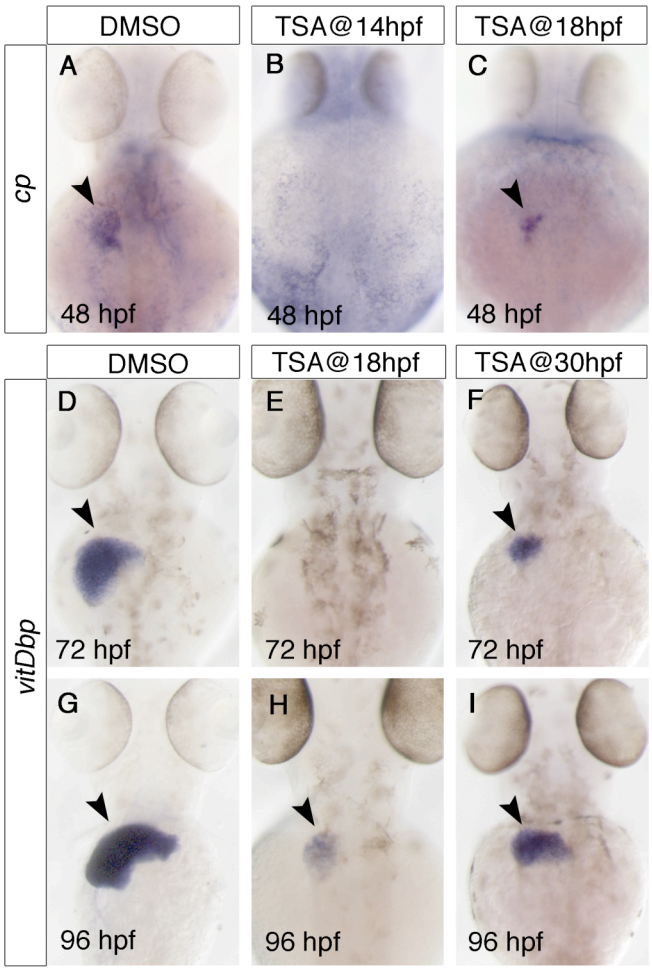


Figure 3.10 Histone deacetylase function is required during late somitogenesis for liver differentiation, and subsequently for organ growth.

Hepatocyte differentiation in TSA treated embryos was assessed by in situ hybridisation for *cp* and *vitDbp* expression. Embryos treated with TSA at 14 hpf showed no *cp* expression at 48 hpf (B). Embryos treated at 18 hpf express *cp* in a reduced domain (arrowhead, C). Embryos treated at 18 hpf showed no *vitDbp* expression at 72 hpf, and only a subset of embryos did at 96 hpf (E, arrowhead H). Conversely, a subset of embryos treated with TSA at 30 hpf expressed *cp* at 72 hpf and 96 hpf (arrowhead, F, I). DMSO was used as a control in all cases (A, D, G), anterior to top.

treated at 18 hpf express *cp*, although in a reduced domain (94%, n=18; Fig. 3.10, compare A, C).

To further elucidate the role of Hdacs in functional hepatic differentiation, I analysed *vitDbp* expression at 72 and 96 hpf. Embryos treated with TSA at 18 hpf failed to express *vitDbp* at 72 hpf, although 63% of embryos showed expression at 96 hpf (n=32, Fig. 3.10, D, H). Interestingly, TSA treatment at 30 hpf resulted in a subset of embryos expressing *vitDbp* at 72 hpf (35%, n=110; Fig. 3.10, F) and similarly to treatment at 18 hpf, 63% of embryos treated with TSA at 30 hpf expressed *vitDbp* at 96 hpf (Fig. 3.10, I). Altogether, this implies that Class I Hdacs are required between 14 and 18 hpf for hepatic specification and that subsequent hepatic differentiation is tightly linked to specification at this time. Morever, Hdacs are required later, between 18 and 30 hpf to drive functional differentiation of hepatocytes. It is likely that Hdacs are required between 14 hpf and 18 hpf to initiate hepatic programs, however this may be separate from a later role after 18 hpf to ensure progressive differentiation. Interestingly, treatment of embryos with TSA at both 18 and 30 hpf resulted in similar numbers of embryos expressing *vitDbp* at 96 hpf. This may reflect that even after 30 hpf Hdacs are required to initiate or maintain hepatocyte differentiation, however this may be partly the result of limitations of the experimental approach used due to lower efficiency of drug penetration at later time points resulting in misleading results. Increased concentrations of DMSO were employed to improve drug absorption, however these proved lethal.

Although treatment with TSA at 18 hpf results in correct timely expression of specification and early differentiation genes, the domain of expression of these genes at 48 hpf is reduced. Treatment of embryos with TSA as late as 30 hpf still results in a small domain of *hhex*, *cp*, and *vitDbp* expression at not only 48 hpf but as late as 96

hpf (Fig. 3.9, 3.10), suggesting that Hdacs play not only a role in timely development of the liver but additionally continue to have a critical function in the growth of the liver. Furthermore, these findings together with earlier *sox17* and *her5* expression data support the fact that the observed defects in *hdac1* mutants are not secondary to defects in early endoderm formation.

3.6 Apoptosis does not account for liver hypoplasia in *hdac1* mutants

In addition to a reduction in number of hepatic progenitors, the reduction in liver size observed in *hdac1* mutants and TSA treated embryos could be the result of decreased proliferation, increased apoptosis, or a combination of these processes. To address the possibility that Hdac1 regulates liver size by promoting cell survival, I examined cell death in *hdac1* mutant and sibling embryos at 30 and 48 hpf using Tunel staining. While changes were observed in other domains of the developing embryo (Fig. 3.11, C, D), no obvious differences in cell death rates were observed in the organ-forming endoderm between *hdac1* mutant and sibling embryos (Fig. 3.11, A, B, E, F), suggesting that liver hypoplasia in *hdac1* mutants at 48 hpf is not the result of increased levels of apoptosis in the liver or organ-forming endoderm.

3.7 Hdac1 function promotes cell proliferation in the endoderm

In *hdac1* mutants or TSA treated embryos liver size is significantly reduced (Fig. 3.1, 3.5, 3.7, 3.10). This reduction is not the result of increased apoptosis, raising the possibility that Hdac1 is required to maintain proliferation in the zebrafish liver. Hdac1 has previously been implicated in regulating cell proliferation at different

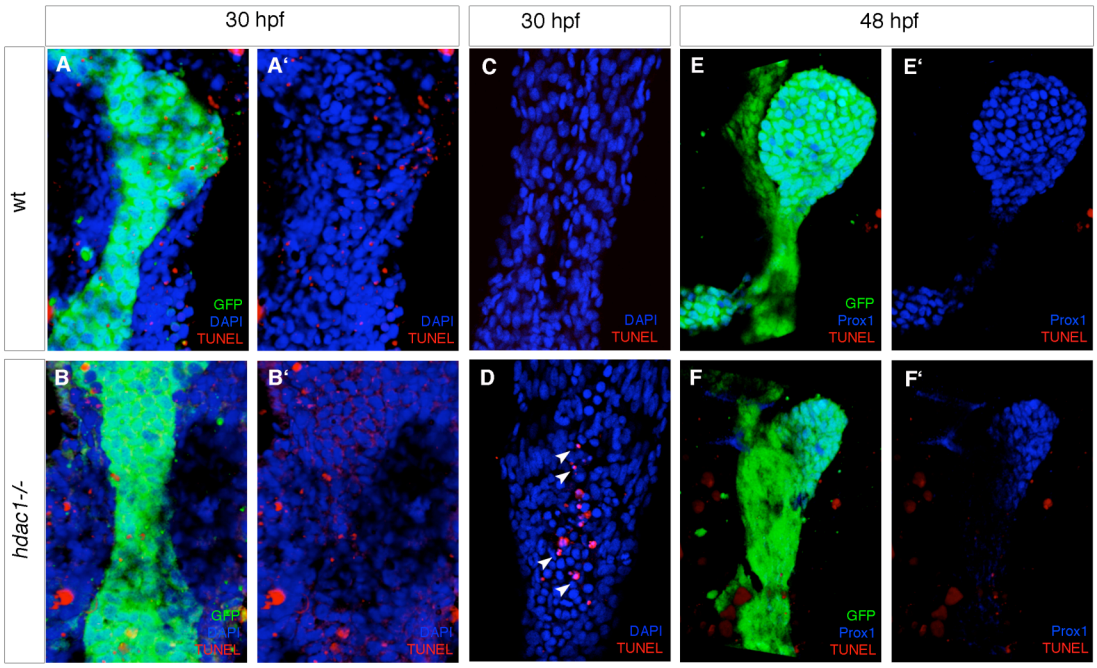


Figure 3.11 Apoptosis does not account for liver hypoplasia in *hdac1* mutants.

(A-B', E-F') Ventral projections of confocal stacks using the *Tg(gutGFP)*^{s854} line stained with Tunel and DAPI (A, B) or α-Prox1 (E, F). Neither wild type or *hdac1* mutants display apoptosis in the liver or organ-forming endoderm at 30 hpf (A-B') or 48 hpf (E-F'). Single sections taken through the neural tube reveal elevated levels of apoptosis in *hdac1* mutants in this tissue when compare to wild type embryos (compare C, D).

stages during mouse and zebrafish development (Cunliffe, 2004; Lagger et al., 2002; Stadler et al., 2005; Yamaguchi et al., 2005). I therefore investigated the role of *hdac1* in cell proliferation in the organ-forming endoderm using α-Phospho-histone H3 (PH3) staining to identify cells undergoing mitosis in sibling and *hdac1* mutant embryos. I observed that the total number of PH3-positive cells was reduced in the *hdac1* mutant organ-forming endoderm between 24 and 48 hpf when compared with sibling endoderm (Fig. 3.12, Table 3.1). Initially, between 24 and 28 hpf, proliferation rates in *hdac1* mutant embryos are slightly reduced when compared with sibling embryos (19.2% and 22.9% reduction, respectively), but are severely reduced at 32 and 48 hpf (47.2% and 48.8% respectively Fig. 3.12, G, H). Importantly, cell proliferation decreases not only in the non-hepatic endoderm, but also in the hepatic endoderm (Table 3.2). At 24 hpf, proliferation in the presumptive hepatic cells could not be scored in *hdac1* mutant embryos as Prox1-positive cells were absent at that stage (Fig. 3.5). Proliferation in hepatoblasts is reduced by 80% and 50% in *hdac1* mutants at 28-32 and 48 hpf, respectively. Notably, the total number of PH3+ cells in the hepatic and non-hepatic endoderm was determined in both sibling and wild type embryos. As the number of proliferating cells as a fraction of total number of hepatic or endodermal cells was not determined, it is possible that the reduction in number of proliferating cells in *hdac1* mutants is due to an overall reduction in number of cells. Determining the ratio of proliferating to total number of cells in the liver and endoderm will determine whether relative proliferation rates are decreased in *hdac1* mutant embryos. Altogether, these data are in agreement with a smaller hepatic domain observed in wild type embryos treated with TSA from 18 hpf onwards (Fig. 3.10). Moreover, it is possible that the reduced number of PH3+ hepatoblasts is the consequence of a smaller hepatic primordium.

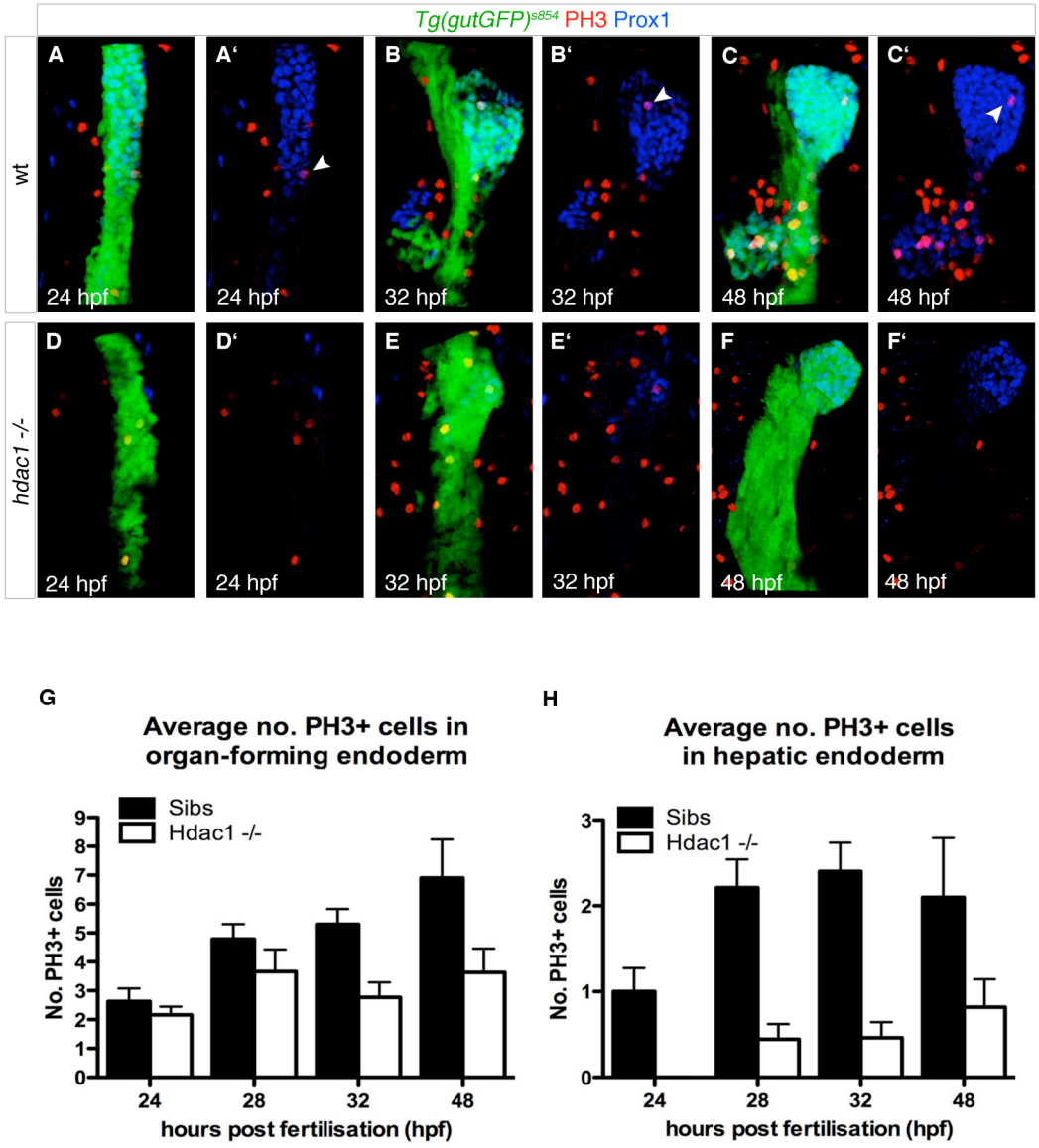


Figure 3.12 Hdac1 promotes cell proliferation in the organ-forming endoderm.

(A-F') Ventral projections showing the *Tg(gutGFP)*^{s854} line, stained for PH3-positive cells (red) and Prox1-positive hepatoblasts (blue) at 24 hpf (A, D), 32 hpf (B, E) and 48 hpf (C, F); dorsal to top. (G-H) *hdac1* mutants display a decreased number of PH3-positive cells in the endoderm including the liver between 24 and 48 hpf. Numbers are supplied in table 1 and 2.

Table 3.1 Average number of PH3+ cells in the organ-forming endoderm

Av. Average, s.d. standard deviation, N. number of embryos, hpf. hours post fertilisation

	hpf	24	28	32	48
Siblings	av.	1	2.21	2.40	2.10
	s.d.	1.10	1.44	1.50	2.18
	N	16	19	20	10
hdac1-/-	av.	0	0.44	0.46	0.82
	s.d.	0	0.53	0.66	1.08
	N	18	9	13	11

Table 3.2 Average number of PH3+ cells in the hepatic endoderm

Av. Average, s.d. standard deviation, N. number of embryos, hpf. hours post fertilisation

	h.p.f	24	28	32	48
Siblings	av.	2.63	4.79	5.30	6.50
	s.d.	1.82	2.25	2.39	4.23
	N	16	19	20	10
hdac1-/-	av.	2.17	3.75	2.77	3.64
	s.d.	1.20	2.29	1.88	2.73
	N	18	9	13	11

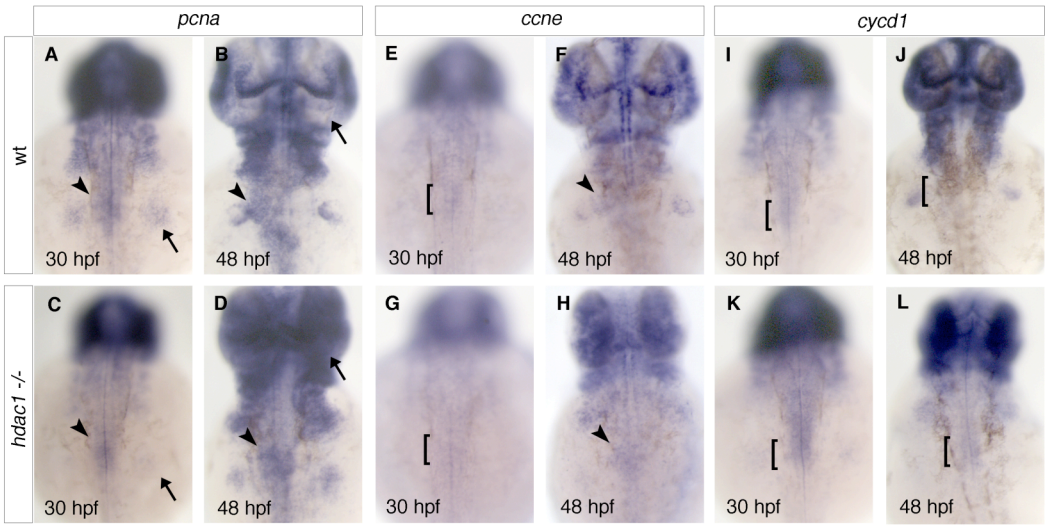


Figure 3.13 Endodermal expression of *pcna, ccne* and *cycd1* is Hdac1-independent

Hdac1 does not control liver proliferation through regulation of *pcna, ccne* or *cycd1* expression. In situ hybridisation analyses of *pcna, ccne* and *cycd1* expression, dorsal views, anterior to top. Wild type embryos express *pcna* in the organ-forming region from around 30 hpf onwards (arrowhead, A, B) and *hdac1* mutants retain this domain of expression (arrowhead, C, D), despite alterations in levels of expression in tissues such as the brain and fin buds (arrow, C, D). *ccne* is expressed at low levels in the organ-forming region from around 30 hpf (brackets, E, G) and at low levels in the hepatic region at 48 hpf (arrowhead, F, H) in both wild type and *hdac1* mutants. Additionally, *cycd1* is expressed in the organ-forming endoderm of wild type and *hdac1* embryos at 30 hpf (arrowhead, I, K), and at low levels at 48 hpf (brackets, J, L).

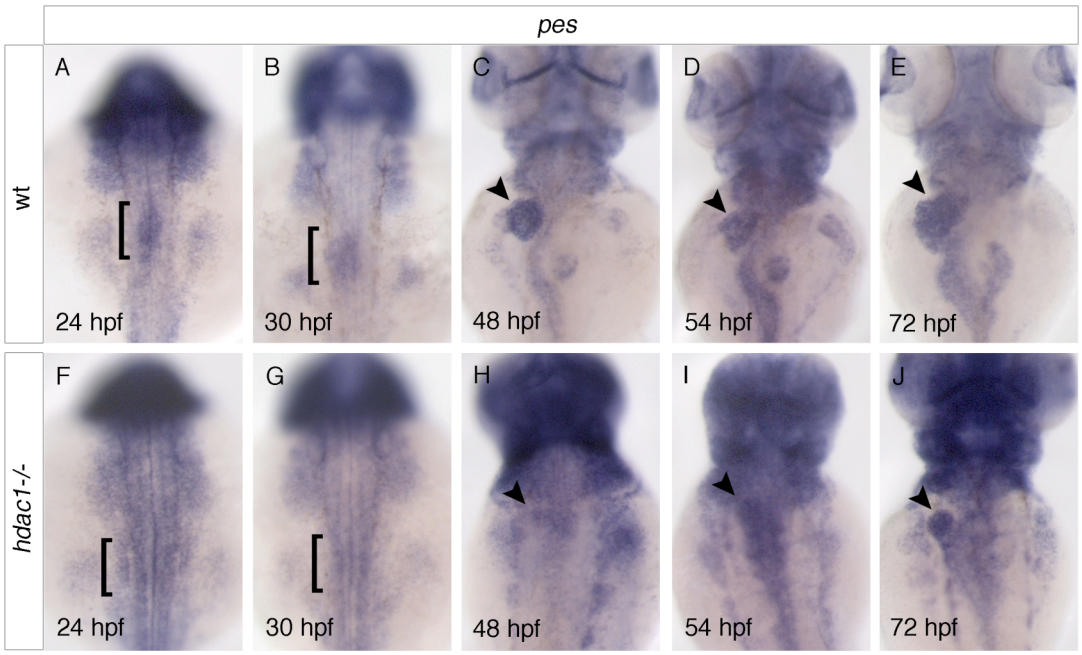


Figure 3.14 Hdac1 is required for early endodermal *pescadillo* expression.

In situ hybridisation analysis of *pes* expression, dorsal views, anterior to top. Wild type embryos express *pes* in the organ-forming region at 24 hpf (bracket, A) and hepatic region from 30 hpf onwards (bracket, B, arrowhead, C-E). *hdac1* mutants lack *pes* expression in the hepatic region at both 24 and 30 hpf (bracket, F, G), however from 48 hpf exhibit wild type levels of *pes* expression (arrowhead, H-J).

To further understand the molecular basis of the proliferation defects observed in *hdac1* mutants, I analysed the expression of a number of cell cycle regulators in wild type and *hdac1* mutants between 24 and 48 hpf, including *pcna, ccne, cycd1, cdk1c* and *p27a*. The majority of genes examined were either not expressed in the organ-forming endoderm at these time points, or no significant differences in levels of expression could be observed between wild type and *hdac1* mutant embryos (Fig. 3.13). However, another candidate for regulating hepatic cell proliferation in *hdac1* mutant embryos is the gene *pescadillo* (*pes;* Allende et al., 1996) which encodes a BRCT-domain containing protein. In wild type embryos, *pes* is expressed in the liver-forming domain at 24 hpf and onwards (Fig. 3.14, A-E). In *hdac1* mutant embryos I failed to observe heightened *pes* expression in the hepatic domain (Fig. 3.14, F, G), despite a mild upregulation of *pes* throughout the trunk of the embryo and a strong upregulation in the head and eyes between 24 and 48 hpf. The latter observations are in agreement with a previously reported increase in cell proliferation in the eye. (Yamaguchi et al., 2005), This confirms that reduced proliferation observed in the hepatic endoderm is not due to a general reduction of proliferation in the entire embryo, indicating that *hdac1* function depends on the tissue context.

Strikingly, *pes* expression levels in the organ-forming endoderm in *hdac1* mutants recover from 48 hpf onwards (Fig. 3.14, H, I). In summary, these data show a requirement for Hdac1 in promoting cell proliferation within the organ-forming endoderm between 24 and 48 hpf, possibly through early regulation of *pes* expression.

3.8 Hdac1 acts cell-autonomously in hepatic development

Factors expressed within both the mesoderm and the endoderm are very important in regulating hepatogenesis. During the initial stages of liver development, *hdac1* is expressed in both endodermal and mesodermal tissues, (Fig. 3.4; Pillai et al., 2004; Yamaguchi et al., 2005). During these stages, *prt/wnt2bb* expression in the LPM appears reduced in *hdac1* mutants (Fig. 3.6), suggesting requirements for Hdac1 within both these tissues to regulate different aspects of organogenesis. To elucidate whether *hdac1* is required cell-autonomously within the endoderm to drive hepatic development, I performed mosaic analysis experiments. To direct wild type cells to an endodermal fate or exclude them from this lineage, I manipulated levels of Casanova/Sox32 (Cas/Sox32) expression, the main endodermal determinant in zebrafish (Alexander and Stainier, 1999). Wild type cells were injected with *cas/sox32* mRNA (Stafford et al., 2006) or *cas/sox32* morpholino oligonucleotides to direct them towards or exclude them from the endodermal lineage, respectively. In addition, fluorescein or rhodamine dextran were coinjected to trace these cells after transplantation in *hdac1* mutant embryos. Labelled wild type cells were subsequently transplanted to the blastoderm margin of *hdac1* mutants at blastula stages, and embryos were allowed to develop until 48 hpf (Fig. 3.15).

Analysis of liver morphology at 48 hpf indicates that wild type cells contributing to the endoderm rescue liver bud morphogenesis (14%, n=11, Fig. 3.16, D). In contrast, small groups of wild type cells contributing to the mesoderm fail to direct wild type-like liver bud formation in the endoderm (0% rescue, n=7; Fig, 3.16, C), however I cannot rule out that the mesodermal clones are too small to cause an apparent rescue. Together, these results suggest a tissue autonomous role for Hdac1 in liver formation.

Further, to determine Hdac1 requirement on a cellular level, I examined *cp* expression in the chimeric embryos, which is expressed in the liver of wild type embryos at 48

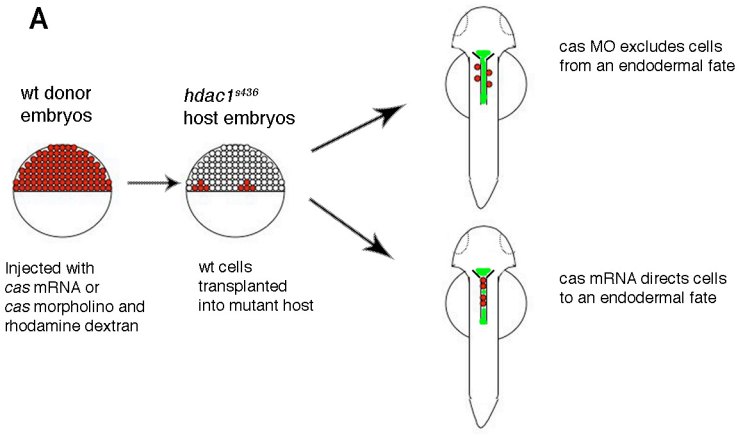


Figure 3.15 Schematic representation of mosaic analysis experiments to determine cell-autonomous requirement of Hdac1.

Wild type donor embryos were injected with either *casanova* morpholino or *casanova* RNA at the one-cell stage to exclude or direct them towards an endodermal fate, in addition to either rhodamine or fluorescein dextran to trace cells post-transplantation. At gastrula stages, cells were removed from the injected wild type embryos, and transplanted to the blastoderm margin of *hdac1* mutants. Embryos were then allowed to develop to 48 hpf, and liver development was analysed. Transplanted cells, red; endoderm, green.

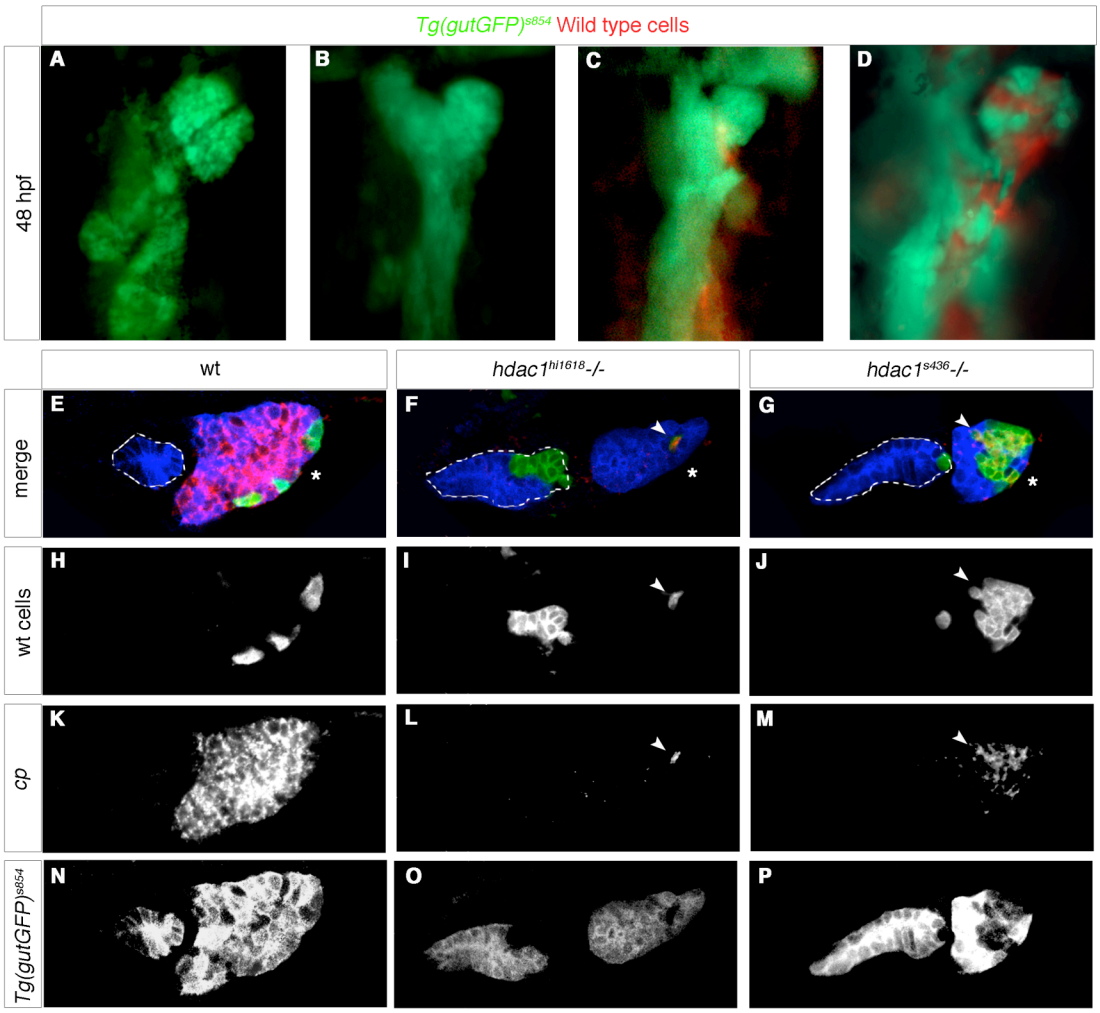


Figure 3.16 Cell-autonomous requirement for Hdac1 in hepatic development.

Ventral view of wild type (A) and *hdac1* mutant (B) organ-forming region at 48 hpf visualised using the *Tg(gutGFP)*^{s854} line. (C, D) Morphological analysis of cell transplantation experiments; wild type cells (red) transplanted into *hdac1* mutants expressing the *Tg(gutGFP)*^{s854} transgene. (C) Wild type cells contributing to the mesoderm fail to rescue liver morphology in *hdac1* mutants. (D) Wild type cells contributing to the endoderm of *hdac1* mutants rescue liver morphology. (E-P) Cell transplantation experiments were analysed by fluorescent in situ hybridisation in conjunction with immunostaining in transverse sections at liver level, using the *Tg(gutGFP)*^{s854} transgene (blue) to highlight the endoderm, *cp* (red) to assess hepatocyte differentiation, and transplanted wild type cells labelled with fluorescein (green). (E, H, K, N) Wild type embryos express *cp* in the liver at 48 hpf. Wild type cells contributing to the hepatic endoderm of *hdac1* mutant embryos express *cp* (arrowhead, F, I, L, G, J, M), however wild type cells contributing to the adjacent digestive tract fail to express

cp (white arrow, F, G). To validate that transplanted host cells injected with *cas* mRNA contribute to the endoderm, *Tg(gutGFP)*^{s854} embryos were used as donors in a subset of transplants (E, N, G, P); due to mild mosaicism of the *Tg(gutGFP)*^{s854} expression, I observe in a subset of transplanted cells very low GFP expression. Asterisks indicate liver, white dashed lines outline the digestive tract.

hpf but not in the liver of *hdac1* mutant embryos at this stage (Fig. 3.6). Analysis of wild type cells contributing to the hepatic endoderm of *hdac1* mutant embryos revealed that these cells express *cp* at 48 hpf (100%, in total n=16, with n=6 for $hdac1^{s436}$ and n=10 for $hdac1^{hi1618}$, respectively; Fig. 3.16, F, G) while the surrounding mutant cells fail to express *cp*. Wild type cells contributing to the liver express *cp* independent of clone size, in small (Fig. 3.16, F, I, L, O) and big clones (Fig. 3.16, G, J, M, P). Wild type cells contributing to the mutant endoderm of the digestive tract are *cp* negative (100%, in total n=24; with n=14 for $hdac1^{s436}$ and n=12 for $hdac1^{hi1618}$ respectively, Fig. 3.16, F, G). These findings indicate a cell-autonomous requirement for *hdac1* within the endoderm in hepatic development.

3.9 Hdac3, but not Hdac8 or 11, may act in concert with Hdac1 in liver organogenesis.

Hdacs are grouped into four classes based on their homology to yeast; Hdac1, 2, 3 and 8 (Class I), Hdac4, 5, 6, 7, 9 and 10 (class II), Sir2-like Hdac (class III), and Hdac11. The latter shares similarity with class I and II enzymes (de Ruijter et al., 2003). Treatment of wild type embryos with the ClassI and II Hdac inhibitor TSA results in a more severe hepatic phenotype than that observed in *hdac1* mutants. This suggests that additional Class I and/or II Histone deacetylases are likely involved in liver organogenesis, acting in parallel, and/or sequentially, as has been suggested for osteoblast differentiation (Westendorf, 2007). Interestingly, loss of Hdac1 in mouse ES cells leads to an increase of Hdac2 and Hdac3 expression, however total Hdac activity was significantly reduced, and upregulation of these alternative Hdacs could not compensate for the loss of Hdac1 activity in cell cycle regulation (Lagger et al., 2002). Furthermore, recent work in zebrafish has suggested that depletion of Hdac3

function leads to defects in liver specification and subsequent differentiation and that loss of both Hdac1 and Hdac3 function leads to more severe hepatic defects (Farooq et al., 2008).

Therefore, I investigated the possibility that additional Class I Hdacs are acting in concert with Hdac1 in promoting hepatic organogenesis. Seven different zebrafish Hdacs are currently described: Hdac 1, 3, (Class I) and 4, 6, 8, 9a and 9b (Class II) (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg). Limited expression data are available, and to date only Hdac3 has been reported to be expressed in the liver (Farooq et al., 2008). In order to determine whether additional Class I and related Hdacs are acting independently of, or in concert with, Hdac1 in liver development, I carried out preliminary experiments using morpholino oligonucleotides to knock down Hdac3, 8 and also the unpublished Class I-related Hdac11 (kindly provided by V. Cunliffe) function separately in wild type and *hdac1* mutants.

First, I examined the expression of these genes at a variety of time-points in wild type and *hdac1* mutants. Hdac3 is expressed at 24 hpf ubiquitously throughout the embryo and no differences could be observed in expression between wild type and *hdac1* mutants (Fig. 3.17, A, D). Expression remains ubiquitous at 30 hpf in wild type embryos (Fig. 3.17, B), although in *hdac1* mutants a mild reduction in level of *hdac3* expression is observed in the fin buds and pharyngeal arches (Fig. 3.17, E). At 48 hpf in wild type embryos, *hdac3* expression becomes restricted to more anterior tissues, with heightened expression in the brain and the fin buds. At this stage, weak expression can be detected in the liver (arrowhead, Fig. 3.17, C). Expression of *hdac3* remained more ubiquitous in *hdac1* mutants at 48 hpf, although, a slight upregulation of *hdac3* expression may be present in the liver, while clear

downregulation of its expression was observed in the fin buds (Fig. 3.17, F). Conversely, *hdac11* was not detected in 24 hpf embryos (data not shown), but was present in wild type and *hdac1* mutants by 30 hpf (Fig. 3.17, G, I). *hdac11* expression could not be detected in the liver region of both wild type and *hdac1* mutant embryos at 48 hpf (brackets, Fig. 3.17, H, J); further, *hdac11* expression could not be detected at 72 hpf, either as the result of unsuccessful experimentation, or *hdac11* is no longer expressed at this time. No *hdac8* probe was available to me, however it is reported to be expressed in a similar pattern to *hdac3* with ubiquitous expression at early stages becoming restricted to anterior structures later in development (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg).

In order to determine the role of Hdac3 in liver formation, I injected 5-10 ng of Hdac3 morpholino oligonucleotides (MO) into the progeny of *hdac1* heterozygote incrosses. Analysis of hepatoblast specification revealed that loss of *hdac3* had no effect upon hepatic *hhex* expression at 24 hpf in sibling embryos (n=47, Fig. 3.18, A, D). In order to assess liver size and morphology at 48 hpf, I performed *foxA3* in situ hybridisations. There appeared to be a small reduction in size of the liver at 48 hpf in *hdac3* morphants. This was accompanied by a reduction in swim bladder and pancreas size, and may be the result of unspecific developmental delay induced by morpholino injection (Fig. 3.18, B, E). Interestingly, the reduction in liver size observed in *hdac1* mutants was not enhanced by morpholino mediated knockdown of Hdac3 (Fig. 3.18, H, K), suggesting that these two genes are not acting together in initial stages of liver budding. Strikingly, injection of *hdac3* morpholino into wild type embryos resulted in wild type expression of *cp* at 48 hpf (data not shown). Further, I analysed liver size at 96 hpf by assessing *cp* expression. Interestingly, a subset of *hdac3* morphants exhibited a reduction in liver size at 4 dpf when compared

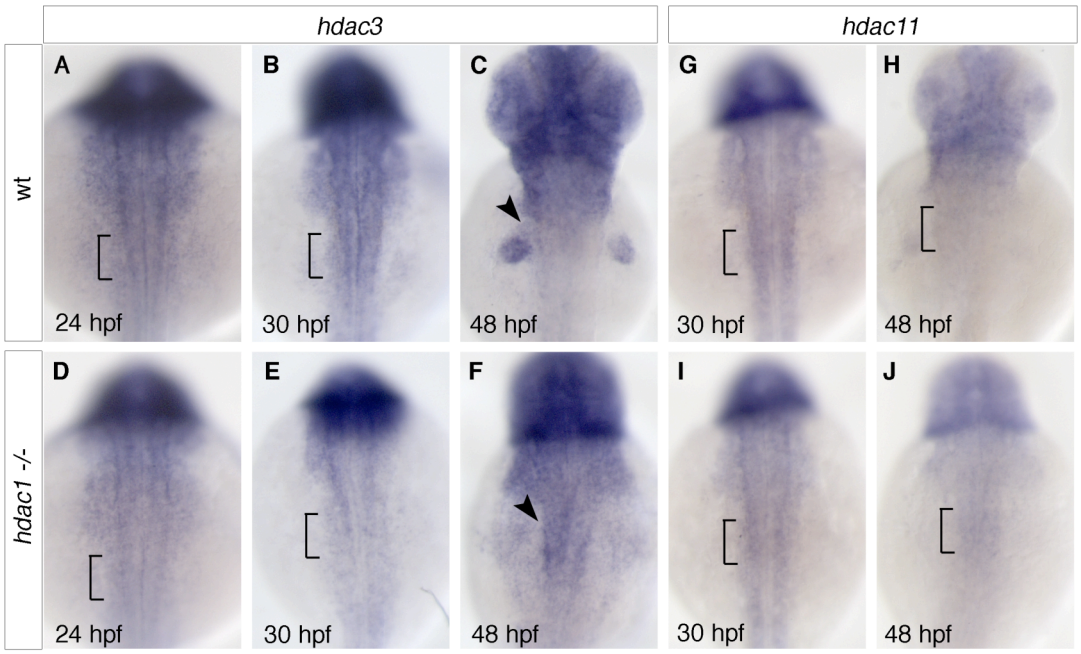


Figure 3.17 Expression of *hdac3* and *hdac11* during liver organogenesis.

In situ hybridisation analyses of *hdac3* and *hdac11* expression in wild type and *hdac1* mutants. Wild type embryos express *hdac3* ubiquitously at 24 hpf at 30 hpf. By 48 hpf expression becomes restricted to anterior structures, including faint expression in the liver (arrowhead, C). *hdac1* mutants exhibit an overall reduction of *hdac3* expression, except for the liver, where a mild upregulation may be observed (arrowhead, F). *hdac11* is expressed in wild type embryos from 30 hpf (G), and becomes restricted to anterior structures at 48 hpf, but expression is not detectable in the liver (bracket, H). *hdac11* expression is unaltered in *hdac1* mutants when compared to wild type (bracket, I, J). Dorsal views, anterior to top, brackets denote organ-forming region.

to wild type embryos (56%, n=34; Fig. 3.18, C, F). Furthermore, injection of *hdac3* MO into *hdac1* mutants enhanced the single knockdown phenotype by a further reduction of *cp* expression at 96 hpf from 50% of *hdac1* mutant embryos expressing *cp* to 20% (n=5; Fig. 3.18, I, L), suggesting that these genes may be acting together in liver growth and differentiation.

Subsequently, I investigated the potential requirement for Hdac8 in hepatic development in wild type and *hdac1* mutant embryos. 5 ng of Hdac8 MO was injected into the progeny of *hdac1* heterozygous incrosses and hepatic specification, endodermal organ morphology, and liver size and differentiation were assessed by *hhex, foxA3* and *cp* expression, respectively, at 24, 48 and 96 hpf. Knockdown of Hdac8 in sibling embryos resulted in wild type *hhex* expression in hepatoblasts at 24 hpf (87%, n=51; Fig. 3.19, A, D). Although a small number of embryos exhibited bilateral hepatic domains of *hhex* expression the incidence of bilateral domains was greatly increased when 10 ng were injected (32% bilateral, n=65; data not shown), and the majority of these embryos died before 48 hpf suggesting bilateral expression of *hhex* could be a non-specific effect of the morpholino. Analysis of hepatic organogenesis at 48 hpf in sibling embryos injected with 5 ng of *hdac8* MO at 48 hpf by *foxA3* expression, revealed that the liver is not reduced in size (86%, n=36; Fig. 3.19, B, E). A small number of morphants exhibited bilateral livers (8%, n=36), and additionally a subset developed smaller livers (5%, n=36). Double knockdown of Hdac1 and Hdac8 resulted in mild defects in digestive system formation - the gut appears slightly wider and more disorganised, and the liver bud smaller, or bilateral (Fig. 3.19, H, K).

Knockdown of Hdac8 does not appear to affect liver size or differentiation in either wild type embryos or *hdac1* mutants at 96 hpf (Fig. 3.19, C, F). Additionally,

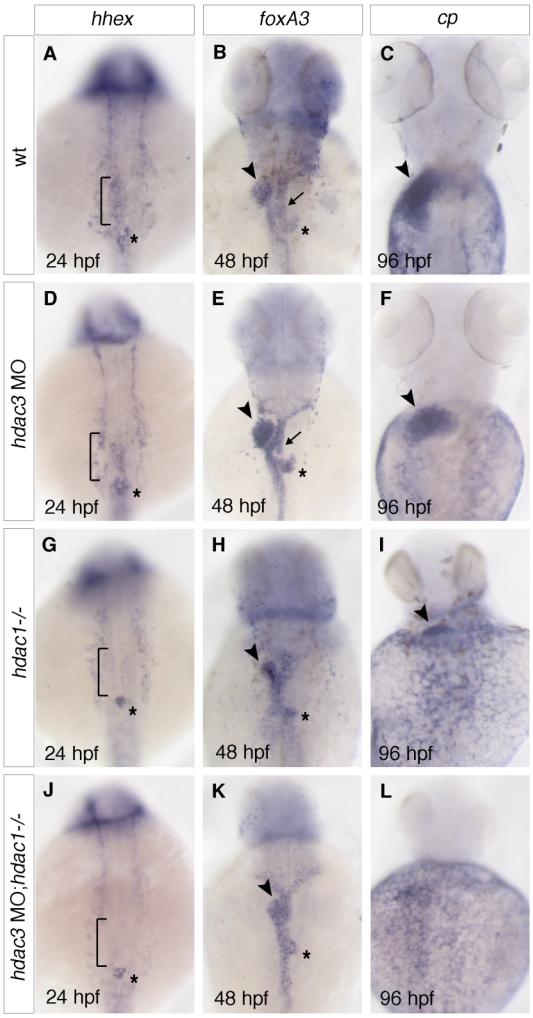


Fig. 3.18 Hdac3 function in liver development

5-10 ng of *hdac3* MO were injected into wild type and *hdac1* mutant embryos and liver specification, morphogenesis, and growth and differentiation were analysed by in situ hybridisation for *hhex*, *foxA3* and *cp* expression, respectively. (A, D, G, J) At 24 hpf wild type embryos express *hhex* in newly specified hepatoblasts, in contrast to *hdac1* mutants (brackets, A, G). *hdac3* morphants express *hhex* in hepatoblasts. (B, E, H, K) Endodermal expression of *foxA3* in wild type embryos allows identification of the liver, pancreas and developing intestine (B), highlighting the reduction in liver size and absence of exocrine pancreas and swim bladder in *hdac1* mutants (H). *hdac3* morphants do not display a reduction in liver size at 48 hpf (arrowhead, E) or exocrine pancreas (asterisk). In addition, double knockdown does not appear to increase the severity of the *hdac1* organogenesis phenotype (compare H, K). (C, F, I, L) *hdac3* morphants display a reduced domain of *cp* expression in the liver when compared to wild type embryos at 96 hpf (compare C, F). The number of *hdac1* mutant embryos expressing *cp* in the liver is reduced upon additional knockdown of *hdac3* (compare I, L).

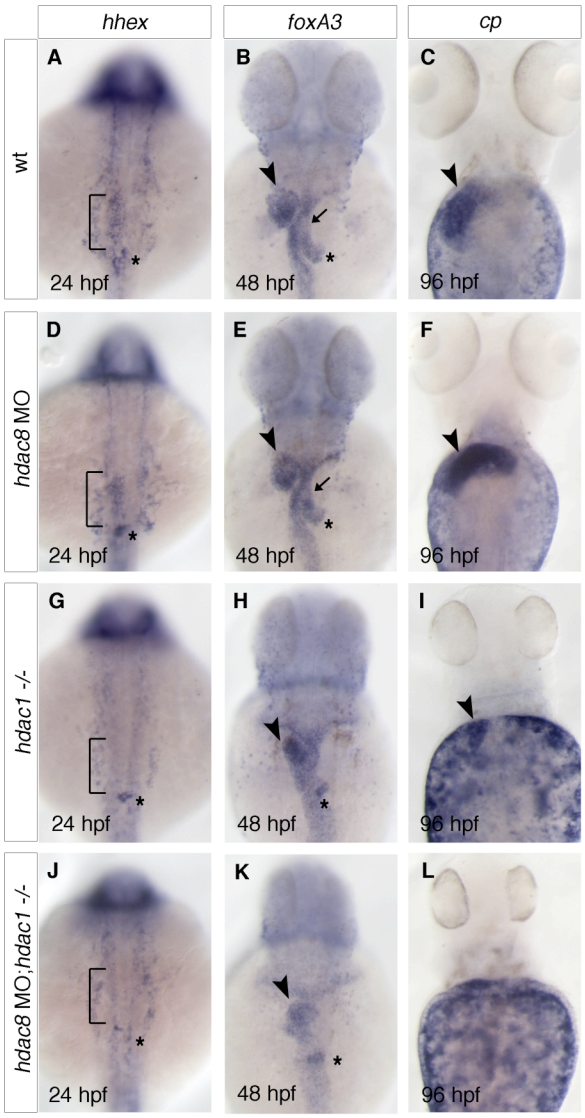


Figure 3.19 Hdac8 is not required for hepatic organogenesis in zebrafish

hdac8 MO was injected into wild type and *hdac1* mutant embryos and liver specification, morphogenesis, and growth and differentiation were analysed by in situ hybridisation for *hhex*, *foxA3* and *cp* expression, respectively. (A, D, G, J) *hdac8* morphants do not display defects in hepatic specification assessed by *hhex* expression when compared to wild type embryos (compare brackets, A, D). (B, E, H, K). Morphogenesis of the liver and pancreas appears wild type in *hdac8* morphants stained for *foxA3* expression (compare arrowheads and arrows, B, E). Double knockdown of *hdac1* and *hdac8* gives rise to slightly more severe morphogenetic defects (compare arrowheads, H, K). (C, F, I, L) *hdac8* morphants do not display defects in liver differentiation and growth, as assessed by *cp* expression (compare arrowheads, C, F). In addition, liver size and number of embryos expressing *cp* is comparable between *hdac1* mutant embryos and *hdac1* mutants injected with *hdac8* morpholino (compare I, L). Asterisks demark pancreas.

depletion of both *hdac8* and *hdac1* does not result in more severe defects in liver differentiation, with 50% of injected mutants expressing *cp* at 96 hpf (Fig. 3.19, I, L). Altogether, this suggests that Hdac8 is not required for liver formation up to 96 hpf, and that Hdac8 is not acting in parallel with Hdac1, or compensating for loss of this gene in *hdac1* mutants.

Finally, I analysed the role of Hdac11, a ClassI-related Hdac, in liver formation by knocking down gene function in both wild type and *hdac1* mutant embryos. Analysis of hepatoblast specification by *hhex* expression at 24 hpf in wild type embryos injected with 5 ng of Hdac11 morpholino revealed that this process occurs normally in the absence of Hdac11 (Fig. 3.20, A, D). Analysis of the morphology of the organ-forming endoderm at 48 hpf reveals that the majority of *hdac11* morphants exhibit no defects in size or morphology of the liver (84%, n=45; Fig. 3.20, B, E). A number of morphants were found to have smaller livers, however these embryos also exhibited characteristics of developmental delay, including swim bladder hypoplasia, suggesting that the smaller liver is due to a general delay rather than knockdown of Hdac11 specifically. Interestingly, the liver in *hdac11* and *hdac1* depleted embryos did not appear more severe than in the *hdac1* mutants alone (Fig. 3.20, H, K). Similarly, analysis of liver size and hepatocyte differentiation at 96 hpf revealed that the majority of *hdac11* morphants have a wild type size liver at this stage (65%, n=34; Fig. 3.20, C, F); a subset of embryos exhibited a smaller liver at this time, although these embryos were generally reduced in size, and the reduction in liver size was not as severe as that observed in *hdac3* morphants, suggesting a continuation of the developmental delay observed at 48 hpf.

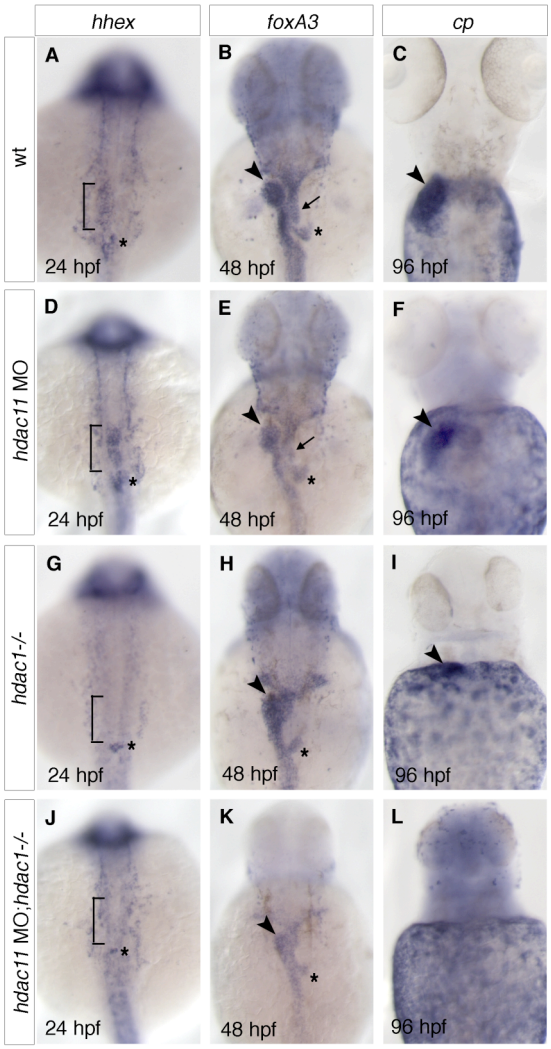


Figure 3.20 Hdac11 is not required for endodermal organogenesis in zebrafish.

hdac11 MO was injected into wild type and *hdac1* mutant embryos, and liver specification, morphogenesis, and growth and differentiation were analysed by in situ hybridisation for *hhex*, *foxA3* and *cp* expression, respectively. (A, D, G, J) *hdac11* morphants do not display defects in hepatic specification assessed by *hhex* expression when compared to wild type embryos (compare brackets, A, D). (B, E, H, K). Morphogenesis of the liver and pancreas appears wild type in *hdac11* morphants when assessed by *foxA3* expression (compare arrowheads and arrows, B, E). Depletion of both *hdac1* and *hdac11* does not give rise to more severe morphogenetic defects (compare arrowheads, H, K). (C, F, I, L) A subset of *hdac11* morphants exhibit a mildly reduced liver when assessed for *cp* expression at 96 hpf (compare arrowheads, C, F). In addition, number of *hdac1* mutants expressing *cp* is reduced upon *hdac11* depletion (compare I, L). Asterisk demarks pancreas.

Double knockdown of *hdac1* and *hdac11* resulted in only 25% of embryos expressing *cp* at 96 hpf (n=8; Fig. 3.20, I, L), suggesting these two genes may be acting together in hepatic differentiation, or that *hdac11* may compensate for the loss of *hdac1*.

Together, these preliminary data suggest that Hdac3, 8, and 11 are not required for hepatic specification in zebrafish. In addition, I propose that Hdac8 is not required for hepatic differentiation or growth. Interestingly, the presented data may point to a role for Hdac3 and potentially Hdac11 in subsequent growth of the liver, and that these genes may compensate for the absence of Hdac1 in hepatic differentiation.

3.10 Endocrine pancreas morphogenesis requires Hdac1 function

In addition to the requirement for Hdac1 in liver formation, early phenotypic analysis has suggested roles for Hdac1 in formation of both the endocrine and exocrine pancreas. To determine whether the requirement for Hdac1 in pancreas development is similar to that described for liver formation, I further examined endocrine and exocrine pancreas formation in *hdac1* mutants.

In zebrafish the endocrine pancreas arises from a loosely connected group of cells positioned along the A-P axis of the organ-forming endoderm at around the level of somites 1 and 2. These cells are specified at around 14 hpf and subsequently aggregate to form a dorsal islet at 24 hpf (Biemar et al., 2001; Field et al., 2003a). Analysis of *hdac1* mutants using the *Tg(gutGFP)*^{s854} line in conjunction with Islet 1/2 staining revealed apparent ectopic groups of endocrine pancreas cells, anterior to the main cluster of the endocrine islet (Fig. 3.5). To confirm this defect in endocrine islet formation, I examined *insulin* expression, which is expressed in the β-cells of the endocrine islet in wild type embryos from around 15 hpf onwards (Fig. 3.21 A-E;

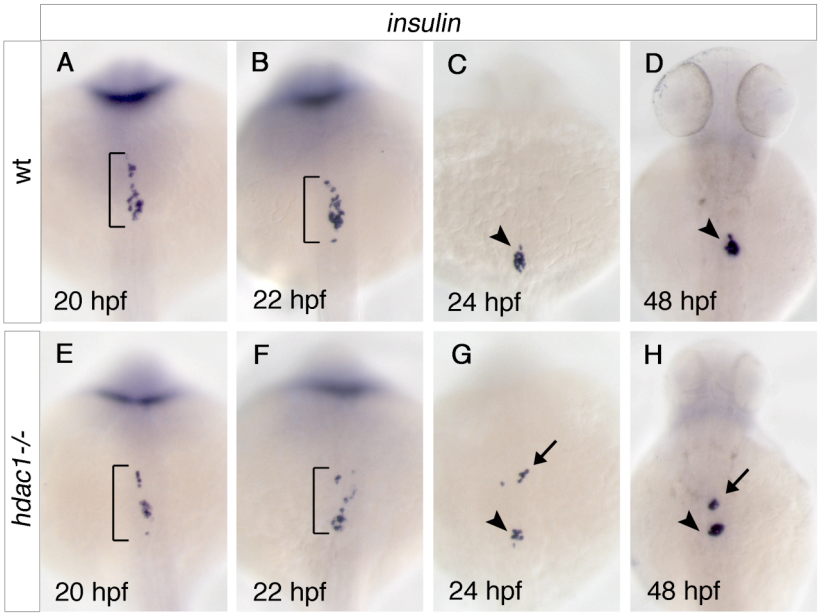


Figure 3.21 Hdac1 is required for endocrine pancreas morphogenesis.

In situ hybridisation analysis of *insulin* expression, dorsal views, anterior to top. In wild type embryos *insulin* expressing cells are spread along the anterior posterior axis of the embryo at 20 hpf (bracket, A). These cells subsequently aggregate posteriorly (B), forming a single endocrine islet by 24 hpf (arrowhead, C, D). *hdac1* mutants exhibit a mild reduction in *insulin* expressing cell number at 20 hpf (bracket E), and localisation of the cells to the posterior domain of the organ-forming region appears impaired (bracket, F), resulting in ectopic anterior clusters of *insulin* expressing cells at 24 and 48 hpf (arrow, G, H).

Biemar et al., 2001). *hdac1* mutants are morphologically indistinguishable from wild type embryos before around 24 hpf; as *hdac1* mutants display defects in cardiac cone formation (data not shown), analysis of *insulin* expression was performed in conjunction with analysis of *cardiac myosin light chain 2 (cmlc2)* expression, allowing identification of *hdac1* mutants at somitogenesis stages. Preliminary analysis suggests a mild reduction in number of *insulin* expressing cells in *hdac1* mutants at 20 and 22 hpf (Fig. 3.21, A, B, E, F). In addition, the cells appear more dispersed along the anteroposterior axis of the organ-forming region in *hdac1* mutants (Fig. 3.21, B, F). *insulin*-expressing endocrine cells aggregate into a single islet at the posterior end of the organ-forming region in wild type embryos at 24 hpf (Fig. 3.21, C). In contrast, in *hdac1* mutant embryos ectopic clusters of *insulin*-expressing cells are observed anterior to the main islet at 24 hpf (Fig. 3.21, G) and are maintained at 48 hpf as revealed by *insulin* and Islet1/2 expression (Fig. 3.21, H, and Fig. 3.4). These were observed in about 61% of *hdac1* mutants, (n=83) and in 20% of phenotypically wild type siblings (n=245) at different stages. This indicates that Hdac1 plays a distinct role in the formation of the endocrine pancreas, likely in a dosage-dependent manner. At this stage I cannot distinguish whether this phenotype is caused by defective migration of endocrine cells or if the ectopic anterior cells have been aberrantly specified at around 24 hpf due to gain of promoting factors or loss of repressive activity within tissues anterior to the endocrine islet.

3.11 Hdac1 is required for exocrine pancreas specification

The pancreas in zebrafish is derived from two anlagen – the dorsal endocrine pancreas and ventral exocrine pancreas; formation of the latter is initiated ventrally around 34 hpf (Biemar et al., 2001; Field et al., 2003a). In addition to the observed defects in

endocrine islet formation in *hdac1* mutants, analysis of exocrine pancreas development using the *Tg(gutGFP)*^{s854} line indicated that the exocrine pancreas failed to initiate at 34 hpf and was not present at 48 hpf (Fig. 3.4). To further understand the defect in exocrine pancreas formation I assessed the expression of two genes. *ptf1a* is the earliest known gene expressed in the forming exocrine pancreas in wild type embryos from around 34 hpf onwards (Lin et al., 2004; Zecchin et al., 2004) while *trypsin*, expressed from around 48 hpf onwards, is a marker of exocrine pancreas function (Biemar et al., 2001). In control embryos at 48 and 72 hpf, *ptf1a* is expressed in the exocrine pancreas (Fig. 3.22, A, B). However, in *hdac1* mutant embryos, expression cannot be detected at 48 hpf (Fig. 3.22, C). At 72 hpf, *ptf1a* is expressed in the majority of *hdac1* mutant embryos, albeit in a reduced and often dysmorphic domain (90%, n=11; Fig. 3.22, D). Similarly, *trypsin* is expressed in the exocrine pancreas at 48 hpf in wild type embryos (Fig. 3.22, E, F), however *trypsin* is first detected in a subset of *hdac1* mutants at around 72 hpf (25%, n=12; Fig. 3.22, G, H). In about 65% of *hdac1* mutants expressing *ptf1a* or *trypsin* in the exocrine pancreas at 72 hpf, the expression domain appears to be placed left of the midline in contrast to expression on the right side of the midline in wild type embryos. Finally, I examined ventral pancreas formation using a transgenic reporter line expressing GFP under the control of the *elastaseA* promoter (Dong et al., 2007). While none of the *hdac1* mutant embryos expressed the reporter at 3 dpf, a subset of embryos were found to express GFP at 4 dpf (Fig. 3.7, T). This further supports the finding that Hdac1 is required for establishment and differentiation of the exocrine pancreas. The lag in pancreas specification differs from the one observed for the liver, as it is initiated only in a subset of embryos about 30-40 hours later than in wild type embryos compared to an approximately 6 hour delay observed for the liver.

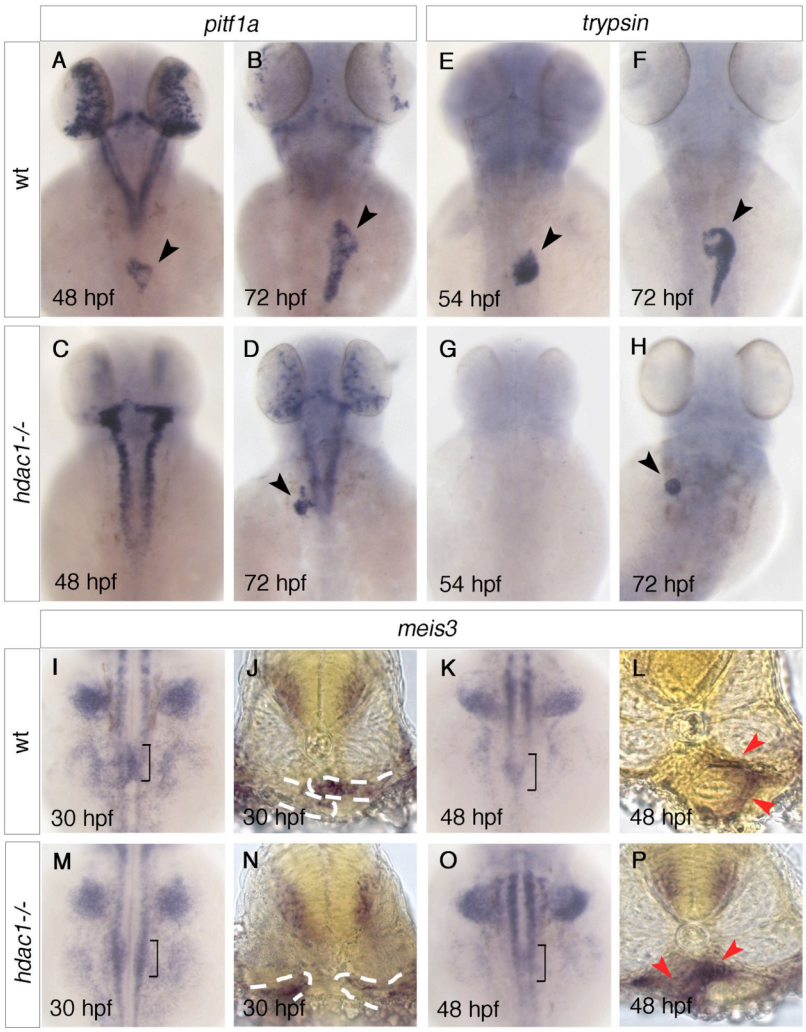


Figure 3.22 Hdac1 is required for exocrine pancreas specification and differentiation.

(A-h) In situ hybridisation analyses of *ptf1a* and *trypsin* expression in wild type and *hdac1* mutants. Wild type embryos express *ptf1a* in the exocrine pancreas from around 34 hpf onwards (arrowhead, A, B). *hdac1* mutant embryos fail to initiate *ptf1a* expression at 48 hpf (C) and it is not detectable until 72 hpf (arrowhead, D). Similarly, *trypsin* is expressed in the exocrine pancreas in wild type embryos at 54 and 72 hpf (arrowhead, E, F), however, *hdac1* mutants fail to initiate *trypsin* expression until 72 hpf (arrowhead H). (I-P) In situ hybridisation analysis of *meis3* expression; I, K, M, O, dorsal views, anterior to top; J, L, N, P, transverse sections through level of pancreas. Wild type embryos express *meis3* medially in the LPM at 30 hpf and 48 hpf (brackets, I, K). *meis3* is expressed bilaterally in the LPM of *hdac1* mutants at 30 hpf (brackets, M, arrowheads, N), however, medial *meis3* expression is observed at 48 hpf in *hdac1* mutants (bracket O, arrowhead, P).

3.12 Hdac1 is required for LPM differentiation and morphogenesis

In zebrafish the LPM adjacent to the organ-forming endoderm provides signals necessary for organ specification and growth, in addition to a requirement in directing digestive tract morphogenesis. Interestingly, embryos depleted of the LPM-derived TALE-box protein Meis3 display strikingly similar pancreatic phenotypes to those observed in *hdac1* mutants, characterised by ectopic anterior clusters of *insulin*-expressing cells (diIorio et al., 2007) in addition to defects in exocrine pancreas formation (Manfroid et al., 2007). This suggests it as a likely candidate to mediate Hdac1 function.

To assess whether loss of *meis3* could account for the pancreatic phenotypes observed in *hdac1* mutants, I examined *meis3* expression in *hdac1* mutant embryos. In wild type embryos, *meis3* is expressed bilaterally in the LPM anterior to the endocrine pancreas and neighbouring the presumptive exocrine pancreas at 24 hpf, and medially as a result of LPM migration from 30 hpf onwards (Fig. 3.22, I,-L; Manfroid et al., 2007). *hdac1* mutants initiate bilateral *meis3* expression in the LPM correctly at 24 hpf (data not shown), suggesting that Hdac1 does not direct endocrine pancreas formation through regulation of *meis3* expression. However, in *hdac1* mutants at 30 hpf *meis3* expression remains in bilateral domains, suggesting a failure of the LPM to migrate medially (Fig. 3.22, M, N). By 48 hpf *meis3* is expressed medially in the majority of *hdac1* mutant embryos (Fig. 3.22, O, P) suggesting subsequent medial migration of the LPM in these embryos. Together, this shows that Hdac1 is not required for initiation or maintenance of *meis3* expression. Nevertheless, the mispositioning of the *meis3*-expressing domain in *hdac1* mutants suggests that Hdac1

is required for correct LPM migration, and that the resulting misplacement of the *meis3* expression domain may result in defects in exocrine pancreas outgrowth.

In addition to the altered *meis3* expression domain in *hdac1* mutants, defects in digestive system looping were observed at a number of stages (Fig. 3.5), a process which is driven by asymmetric movements of the LPM (Horne-Badovinac et al., 2003), further supporting a role for Hdac1 in LPM migration. To determine whether alternative tissues ectopically overexpress *meis3*, or whether defective LPM migration could account for the altered *meis3* expression pattern and defective digestive tract looping, I examined LPM morphology in *hdac1* mutants.

In wild type embryos, the LPM is formed of two bilateral sheets at 24 hpf, flanking the organ-forming endoderm. Between 24 and 30 hpf the left LPM migrates dorsal to the gut across the midline, while the right LPM moves down ventrally resulting in an asymmetric positioning of both lateral plates at 30 hpf (Fig. 3.23, A, B). I observed in transverse sections through the organ-forming region of *hdac1* mutant embryos that both the left and the right LPM failed to move medially at 30 hpf (n=30 Fig. 3.23, C, D), and that the organisation of this tissue appears disrupted, with the left lateral plate appearing more severely affected. The two sheets of the lateral plate no longer exhibit a characteristic squamous and columnar cell polarisation, and instead are made up of cuboidal cells, suggesting a defect in establishment of epithelial polarity (Fig. 3.23, C, D). Interestingly, in a subset of embryos, Prox1-positive hepatoblasts are present on the left side of the endodermal rod, suggesting that migration of the LPM is not essential for directed outgrowth of the liver (Fig. 3.23, C, D). In summary, these findings suggest that misplaced *meis3* expression and the mild gut-looping defects in *hdac1* mutants are likely due to LPM differentiation and migration defects.

3.13 Hdac1 promotes hepatic and exocrine pancreatic primordia formation from foregut tissue.

Concomitant to the significantly reduced liver bud and absence of exocrine pancreatic tissue in *hdac1* mutants at 48 hpf, I observed an increase of foregut tissue in the domain these organs originate from (Fig. 3.5). To examine the possibility that defects in hepatic and exocrine pancreatic primordia formation leads to an expansion of alimentary canal tissue within the foregut domain, I determined the number of hepatic, pancreatic and foregut cells in sections of the organ-forming region at four stages between 28 and 48 hpf in *hdac1* mutant and sibling embryos (Fig. 3.24, A-D). Consistent with my previous results I observed a strong reduction of hepatic cell numbers and an absence of exocrine pancreatic tissue in *hdac1* mutants at the stages examined (Fig. 3.25, B). Comparing the number of foregut cells to the total number of endodermal cells in a section in mutant and sibling embryos, I observed a 21.2 to 31% increase of foregut endodermal cells in *hdac1* mutant embryos between 28 and 48 hpf (wt siblings: 28 hpf=58.9%, 30 hpf=53.3%, 40 hpf=48.1%, 48 hpf=46.3%; *hdac1* mutants; 28 hpf=86.8%, 30 hpf=84.2%, 40 hpf=69.3%, 48 hpf=70.2%; Fig. 3.25, A). These changes are statistically significant: 28 hpf $p=0.011$, 30 hpf $p=0.0002$, 40 hpf $p<0.0001$, 48 hpf $p=0.001$. These findings suggest that Hdac1 is required for timely formation of the hepatic and pancreatic primordia from the foregut endoderm and in the absence of *hdac1* function, liver and pancreas formation are not only delayed, but in addition foregut tissue forms at the expense of the developing liver and pancreas, resulting in an increase of nonhepatic foregut endoderm.

Furthermore, these experiments have revealed an overall reduction of endodermal cells in the organ-forming region in *hdac1* mutants between 28 hpf and 48 hpf (28

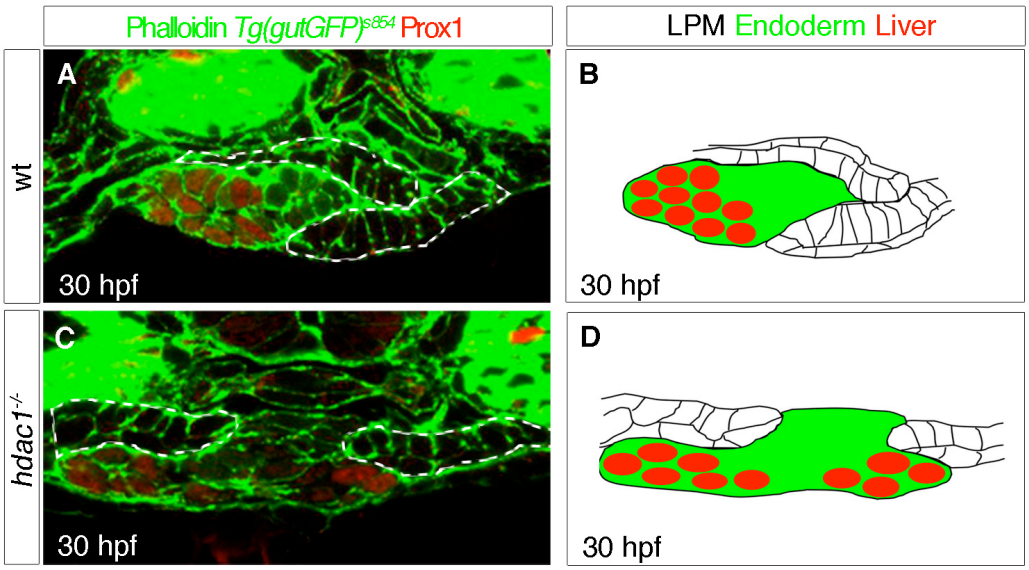


Figure 3.23 Hdac1 is required for correct LPM migration and morphology.

(A,C) Transverse sections at the liver level of *Tg(gutGFP)*^{s854} wild type and *hdac1* mutant embryos, stained with Phalloidin (green) highlighting β-Actin, and α-Prox1 (red) denoting hepatoblasts. In wild type embryos at 30 hpf the LPM consists of two polarised epithelia that have migrated asymmetrically, with the left lateral plate migrating dorsally to the endoderm and the right lateral plate migrating ventrolaterally. At this time, the plates are each composed of two sheets of epithelia, one made of columnar cells and the other of squamous cells (B). In *hdac1* mutants the LPM remain in a lateral position, failing to undergo medial migration (C). Additionally, the epithelial organisation of this tissue is altered; the LPM in *hdac1* mutants contains only cuboidal cells, while in wild type embryos it is comprised of both squamous and columnar epithelial cells. (compare B and D).

hpf=21.5%, 30 hpf=22.3%, 40 hpf=19.9%, 48 hpf=30%; Fig. 3.25, B), confirming the results of the proliferation studies (Fig. 3.13).

In addition to hepatic and pancreatic differentiation, the non-hepatic foregut undergoes differentiation during embryogenesis. Ex vivo studies in mouse intestinal cells has suggested that Class I Hdacs (specifically Hdac1 and Hdac2) must be downregulated to allow correct intestinal epithelial differentiation to occur (Tou et al., 2004). Similarly, work in colon cancer cell lines has suggested that Class I Hdacs, in particular Hdac3, are involved in repressing intestinal differentiation through regulation of levels of the cell-cycle regulator p21 (Wilson et al., 2006).

The implication of Hdacs in regulation of intestinal differentiation, and the observed increase in foregut tissue led me to determine whether *hdac1* mutants exhibit enhanced intestinal differentiation, and whether the increase in foregut cells results in disruption of gut architecture and lumen formation. *vhnf1*, a MODY-5 family member, and *claudin15* are expressed in the differentiating intestine and have been implicated in lumen formation in zebrafish (Bagnat et al., 2007). Wild type embryos express *vhnf1* in the endoderm posterior to the liver at 30 hpf, and 48 hpf (Fig. 3.26, A, B); in *hdac1* mutant embryos at 30 hpf a mild reduction in *vhnf1* expression was observed (Fig. 3.26, C). However, no changes in endodermal expression of *vhnf1* were present at 48 hpf (Fig. 3.26, D). Interestingly, *hdac1* mutants did display an upregulation of *vhnf1* expression in the pronephric tubules at both stages (Fig. 3.26, C, D). Furthermore, analysis between 36 and 48 hpf revealed that the onset of *claudin15* expression is mildly delayed, and the expression weaker at 48 hpf in *hdac1* mutant embryos when compared to wild type siblings. (0%, n=10 for 36 hpf, 91%, n=33 for 48 hpf; Fig. 3.26, E, F, H, I). At 72 hpf the gut appears thinner in *hdac1* mutant embryos when compared to wild type siblings (n=17; Fig. 3.26, G, J),

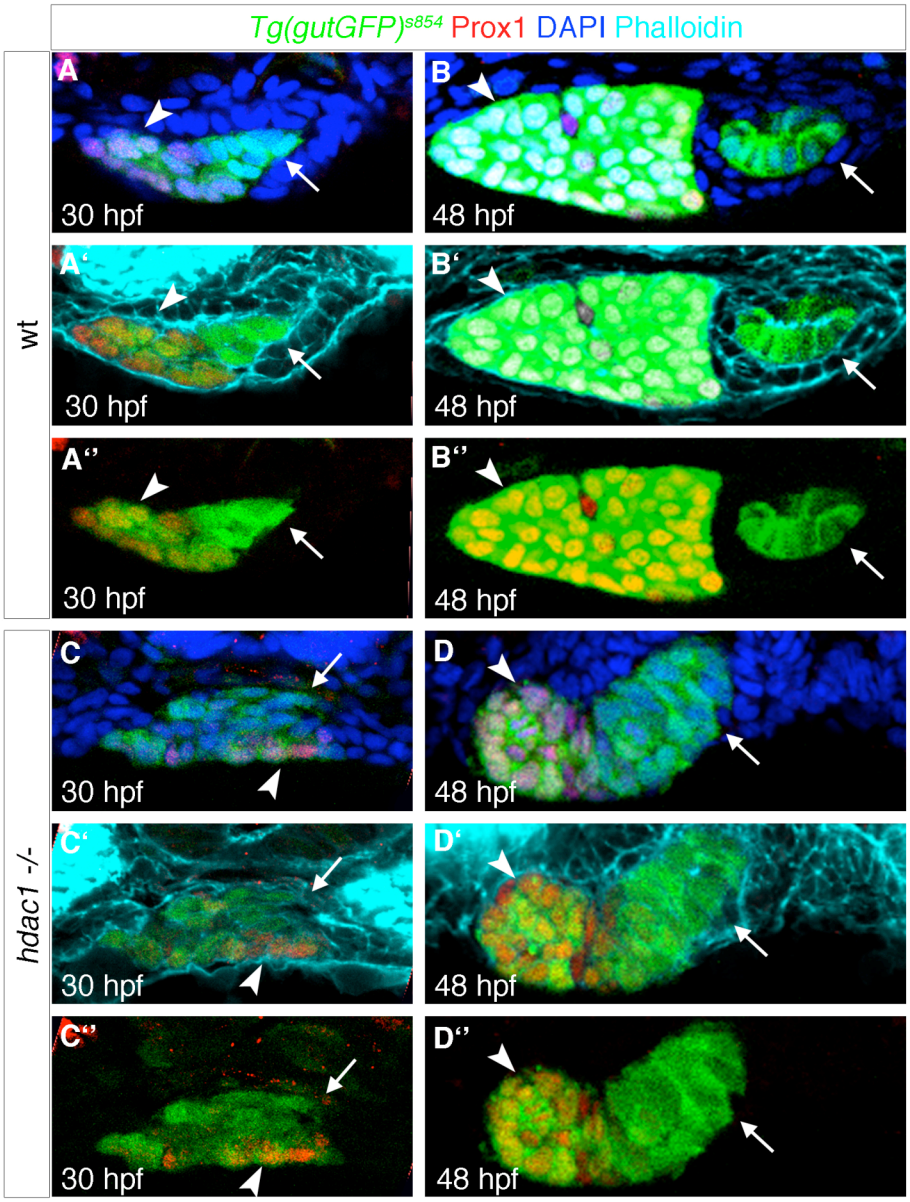
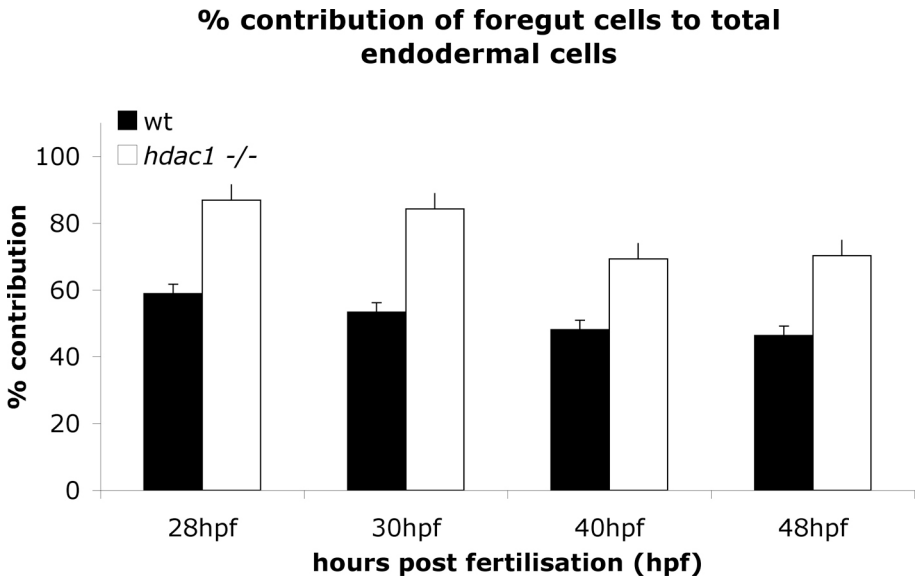


Figure 3.24 Hdac1 promotes liver and exocrine pancreas specification at the expense of foregut tissue.

Transverse sections at the level of the organ-forming region in wild type (A, B) and *hdac1* (C, D) mutant embryos using the *Tg(gutGFP)*^{s854} transgene (green), stained with DAPI (blue), Phalloidin (cyan) and α-Prox1 (red) to highlight hepatic and non-hepatic foregut endoderm and facilitate cell counting. Arrowhead, liver; arrow, intestine.

A



B

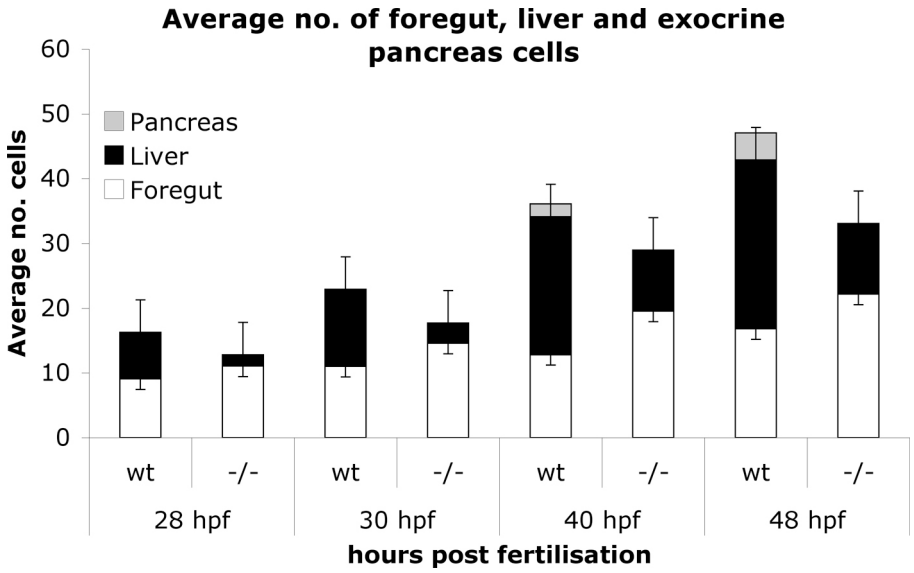


Figure 3.25 Hdac1 promotes hepatic and pancreatic specification at the expense of the foregut.

(A) % Contribution of non-hepatic foregut cells to total endodermal cells in wild type and *hdac1* mutants. (B) Average number of foregut, liver and exocrine pancreas cells in wild type and *hdac1* mutants.

consistent with our observations in the *Tg(gutGFP)*^{s854} line at this and later stages.. Taken together my data demonstrate that Hdac1 is required to promote organ fate from the neighbouring foregut tissue. However, in the absence of Hdac1, the increase in foregut tissue does not result in increased differentiation.

The thinner alimentary canal observed in *hdac1* mutants at 72 hpf may be the result of a failure in lumen formation and expansion. Due to the delay in *vhnf1* and *claudin15* expression in *hdac1* mutants this seems a likely possibility. Therefore, to determine the effect of decreased foregut differentiation and increased foregut tissue on gut morphology, I examined gut organisation and lumen morphogenesis in *hdac1* mutant embryos. I analysed transverse sections of the foregut in *hdac1* mutant and sibling embryos at a variety of stages between 48 hpf and 4 dpf, using the *Tg(gutGFP)*^{s854} transgene, stained with either Phalloidin and α-Prox1, or α-HNF4 (highlighting the liver and foregut) and α-Laminin (expressed in basal membranes). At 48 hpf in wild type embryos, the intestine is comprised of a single layer of cells forming a tubular structure, and a single focal lumen has formed but has yet to expand (Fig. 3.27, A). In contrast, in the majority of *hdac1* mutants the increased numbers of intestinal cells are highly disorganised, failing to form a tubular structure, and a single focal lumen cannot be distinguished (Fig. 3.27, B, C, D). By 72 hpf, lumen expansion has occurred in wild type embryos (Fig. 3.27, E). In contrast, *hdac1* mutants display a variety of phenotypes at 72 hpf, which are more pronounced at 96 hpf; a subset of embryos form 2 or more foci which have failed to undergo expansion (Fig. 3.27, F, L), while a further subset exhibit several lumens (Fig. 3.27, G, J). A small subset of *hdac1* mutant embryos undergo formation of a single lumen (Fig. 3.27, H, K), which appears to begin expansion.

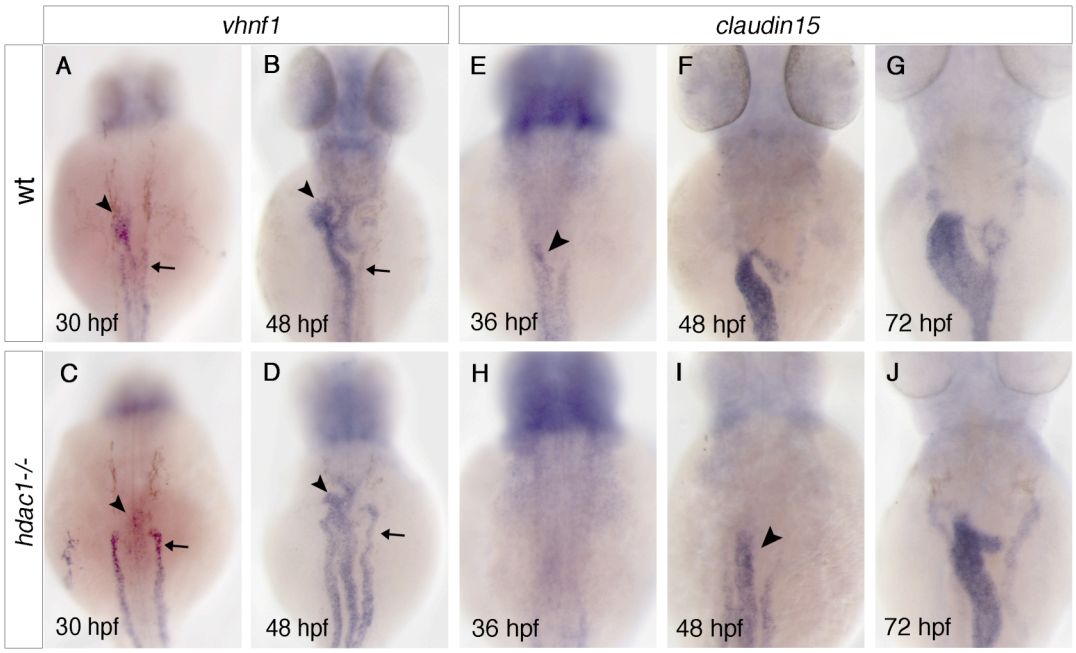


Figure 3.26 Hdac1 is required for digestive tract differentiation in zebrafish

In situ hybridisation analyses of *vhnf1* and *claudin15* expression, dorsal views, anterior to top. (A-D) Wild type embryos express *vhnf1* in the liver and foregut endoderm at 30 hpf (arrowhead, A) and the endoderm posterior to the pharyngeal arches at 48 hpf (B). *hdac1* mutants exhibit a slight reduction in *vhnf1* expression in the endoderm at 30 hpf (arrowhead, C); subsequently levels of expression are comparable with wild type at 48 hpf (arrowhead, D). Additionally, *hdac1* mutants show upregulated *vhnf1* expression in the pronephros at all stages examined (arrows, C, D). (E-J) Wild type embryos initiate *claudin15* expression in the endoderm posterior to the liver at 36 hpf (arrowhead, E), and expression is maintained at 48 and 72 hpf (F, G). In contrast, *claudin15* expression is absent in *hdac1* mutants at 36 hpf (H) but is initiated by 48 hpf (arrowhead, I) and maintained at 72 hpf (J).

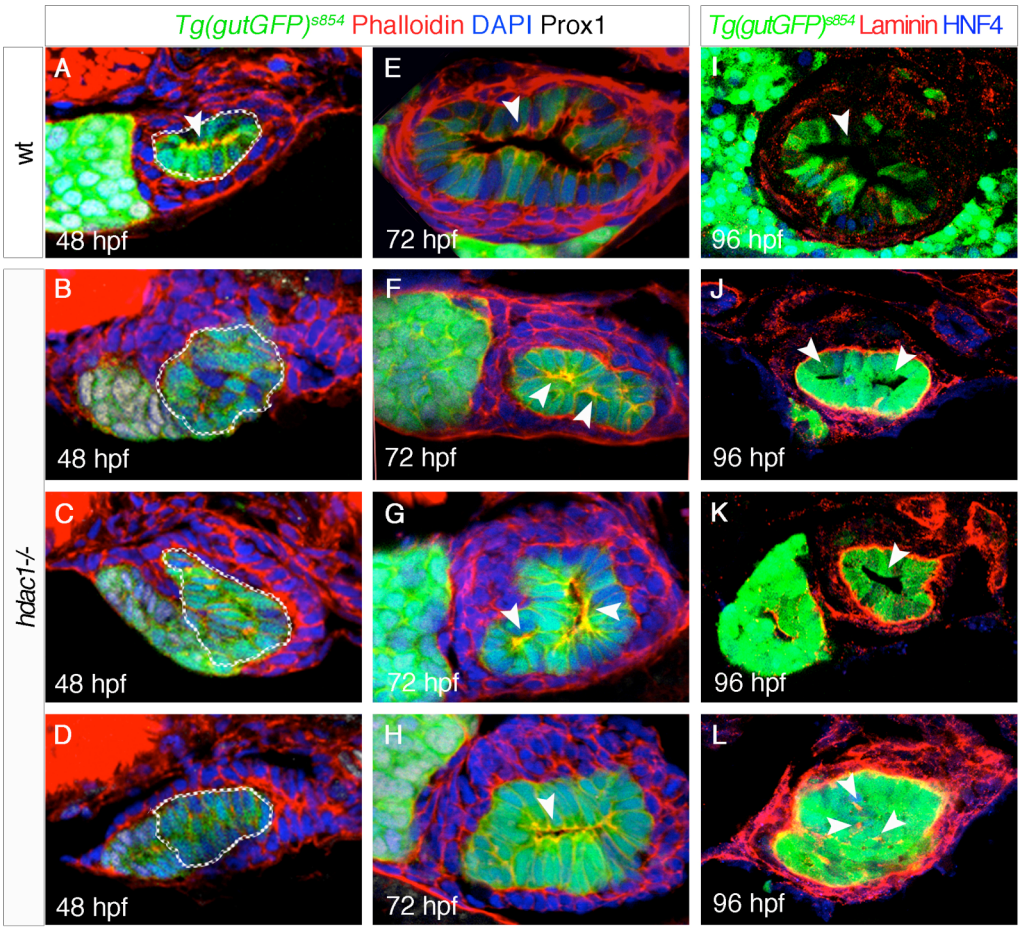


Figure 3.27 Hdac1 is required for digestive tract lumen formation in zebrafish.

Transverse sections through *Tg(gutGFP)*^{s854} wild type and *hdac1* mutant embryos at liver level, stained with (A-H) Phalloidin (red), DAPI (blue) and α-Prox1 (white) or (I-L) α-Laminin (red) and α-HNF4 (blue). At 48 hpf, wild type embryos form a single lumen (A), which subsequently expands (E, I). *hdac1* mutant embryos display a variety of lumen formation defects. At 48 hpf, the majority of *hdac1* mutants display a disorganised foregut endoderm (B-D). At 72 and 96 hpf, a subset of embryos form multiple, unexpanded lumens (arrowheads, F, L), while a subset with multiple lumens begin to undergo expansion (arrowheads, G, J). A small number of *hdac1* mutant embryos have only a single lumen (arrowhead, H, K). (A-D) Dotted line, intestine.

As Hdac1 has been suggested to regulate the expression of ECM components, it is interesting that *hdac1* mutant embryos analysed at 96 hpf appear to have increased amounts of Laminin surrounding the intestine when compared to wild type embryos (Fig. 3.27, compare J, K, L with I). The presence of Laminin suggests that establishment of basal identity within the cells of the digestive tract has occurred normally although further analyses will be required to determine whether correct apical polarity, required for formation of tight junctions and subsequent lumen formation, has been established. The role of Laminin in expansion of the digestive tract remains unclear, and it will be interesting to probe this further to characterise the expression of ECM components in *hdac1* mutants to further understand the failure in single lumen formation.

3.14 Discussion

Complex multi-organ systems such as the digestive system, in which the liver, exocrine and endocrine pancreas arise as distinct anlagen from the foregut endoderm, demand tightly coordinated steps of patterning and morphogenesis to allow formation of each organ. As we are beginning to identify the roles of several transcription factors within these processes we need to understand how their activities are regulated. Chromatin remodelling, mediated by levels of DNA methylation and histone acetylation, is important for controlling the access of regulatory elements to their target DNA sequences. Although the importance of Hdacs in gene regulation is well established, and their specific roles in differentiation of embryonic stem cells, as

well as hepatic and pancreatic cancer are emerging (Glozak and Seto, 2007), their specific roles in embryonic development, and in particular digestive system development, are still poorly understood. In mice, depletion of Hdac1 in the entire embryo leads to widespread proliferation defects during gastrulation and early lethality that are at least partly due to up-regulation of the cell-cycle inhibitor p21 (Lagger et al., 2002). Therefore, examining Hdac1 function in later events such as liver organogenesis in mouse would require conditional tissue specific approaches. In contrast, zebrafish *hdac1* mutant embryos pass through gastrulation exhibiting mild patterning defects in a subset of tissue, but without severe early morphological defects because of maternal contribution (Nambiar and Henion, 2004; Nambiar et al., 2007). Thus, Hdac1 dependent processes occurring at later stages of embryonic development can be examined, such as neurogenesis, eye or fin development (Cunliffe and Casaccia-Bonnefil, 2006; Stadler et al., 2005; Yamaguchi et al., 2005). My genetic studies revealed that the broadly expressed chromatin remodelling factor Hdac1 has specific roles in liver as well as pancreatic organogenesis in zebrafish. This establishes zebrafish as a highly suitable model organism for elucidating the role(s) of Hdacs in endodermal organogenesis.

Specificity of Hdac1 in endodermal organogenesis

I have shown specific roles for Hdac1 in liver specification, and subsequent differentiation and growth. Further analysis of *hdac1* mutants has revealed that in addition to liver defects, Hdac1 is required for very distinct aspects of pancreas formation, and the promotion of hepatic and pancreatic organogenesis from foregut tissue, resulting in disrupted gut architecture. Therefore, as multiple endodermal structures are affected in *hdac1* mutants, coupled with the early ubiquitous expression

of *hdac1*, this raises the question of whether the observed defects in liver formation in *hdac1* mutants are specific or secondary to general defects in early endoderm formation. Three lines of evidence support the view that Hdac1 carries out distinct functions. First, I detect no obvious changes in early endodermal *sox17, her5* and *foxA1* expression during late gastrulation stages and at 24 hpf, respectively. *hdac1* is ubiquitously expressed from the 1-cell stage until 18 hpf (Cunliffe and Casaccia-Bonnefil, 2006; Nambiar et al., 2007; Pillai et al., 2004; Yamaguchi et al., 2005), suggesting maternal contribution of wild type Hdac1 may allow early endodermal development to proceed undisrupted in *hdac1* mutants. Second, inhibition of Hdac activity between 14 hpf and 18 hpf by application of TSA leads to a severe or complete loss of hepatoblast specification, confirming that this defect is specific to Hdac1 function at this stage and not secondary to earlier defects in endoderm development. Concomitantly, I found that expression of *prt/wnt2bb*, known to promote hepatoblast formation, is absent under these conditions. Thirdly, the organs developing from the ventral foregut are differently affected. Hdac1 function appears to be required for timely liver and exocrine pancreas specification, differentiation and growth, in contrast to a requirement in endocrine pancreas morphogenesis, implying that Hdac1 is playing distinct roles in different aspects of endodermal organogenesis. Despite its relatively broad expression pattern, Hdac1 has already been shown to play tissue specific roles in various aspects of embryonic development in *C. elegans, Drosophila*, zebrafish and mice (Calvo et al., 2001; Cunliffe and Casaccia-Bonnefil, 2006; de Ruijter et al., 2003; Dufourcq et al., 2002; Lagger et al., 2002; Mannervik and Levine, 1999; Pillai et al., 2004; Yamaguchi et al., 2005). Similarly, depletion of the chromatin remodelling factors DNA methyltransferase I and/or H3K9 histone methyltransferase Suv39h1 in embryos leads to organ specific defects in terminal

differentiation of the intestine, exocrine pancreas and retina (Rai et al., 2007; Rai et al., 2006), indicating that broadly expressed transcriptional modifiers regulate distinct processes of development.

Hdac1 functions in hepatic organogenesis

My genetic study indicates that *hdac1* is required for the timely onset of hepatoblast specification and their subsequent differentiation. Analysis of *hhex* and Prox1 expression in *hdac1* mutant embryos and embryos in which Hdac function was blocked by application of TSA, revealed defects in the onset of hepatoblast formation. Similar defects in early liver development were observed by timed inhibition of Hdac activity (preferentially class I) using Valproic acid (Farooq et al., 2008). In *hdac1* mutant embryos, *prt/wnt2bb* expression in the LPM is reduced, possibly resulting in the delay in hepatoblast formation. Moreover, inhibition of Hdac activity by TSA treatment revealed a requirement for Hdac function before 18 hpf, which coincides with *prt/wnt2bb* expression in the LPM (Ober et al., 2006). Together, this supports the view that decreased *prt/wnt2bb* levels are contributing significantly to the defect in hepatoblast formation in Hdac1 depleted embryos. Hdac1 activity may regulate – directly or indirectly – levels of *prt/wnt2bb* expression in the LPM. Importantly, the distance between the bilateral *prt/wnt2bb* expression domains in the LPM appears unaltered when compared to wild type at the time of hepatoblast formation, despite the observed LPM migration defects at later stages, indicating that LPM migration defects occur at subsequent stages. It is intriguing to speculate that Hdac1 controls signals from the endoderm that are required to promote *prt/wnt2bb* expression in the mesoderm, similar to the role of the endoderm in promoting heart development (Foley et al., 2006).

It is interesting to speculate that hepatoblast specification occurs once threshold levels of inductive signals have been reached. It is possible that potential hepatoblasts are only capable of responding to threshold levels of mesodermally derived inducing factors and since *hdac1* mutants exhibit reduced levels of *prt/wnt2bb* it may take longer for the threshold required for specification to be reached, resulting in the observed delay in specification. The same scenario may be applied to differentiation, where certain thresholds of genes expressed in hepatoblasts are required for hepatocytes and biliary cells to undergo differentiation, as has been demonstrated for the requirement of HNF6 in driving hepatocyte maturation in mouse (Beaudry et al., 2006).

It has been shown that additional factors to *prt/wnt2bb* are required in the LPM to promote hepatoblast specification, such as Fgfs and Bmps (Chung et al., 2008; Shin et al., 2007). Since liver specification still occurs in *hdac1* mutants, and the hepatic bud at 48 hpf is larger in *hdac1* mutants than in *prt/wnt2bb* mutants (Ober et al., 2006), this raises a number of possibilities. *Hdac1* mutants exhibit reduced, not absent, *prt/wnt2bb* expression, therefore this may explain the milder hepatic phenotype. Notably, studies in amniotes and zebrafish have demonstrated that Fgfs and Bmps expressed in the LPM are required for hepatic specification, likely acting in parallel to Wnt signalling (Calmont et al., 2006; Deutsch et al., 2001)(Jung et al., 1999; Shin et al., 2007; Zhang et al., 2004). Therefore it is possible that other factors, such as Fgfs and/or Bmps are able to partially compensate for the reduction in Wnt signalling in *hdac1* mutants, to promote hepatic specification.

Further, it has been shown that Hdac1 interacts with Rerea (a member of the Atrophin family of transcriptional regulators) and *fgf8* to regulate multiple processes of embryonic development, and is capable of interacting solely with Fgfs to regulate

expression of Fgf target genes (Plaster et al., 2007). In addition, studies have shown that Fgf8 is specifically required for liver outgrowth (Jung et al., 1999). Therefore, Hdac1 may be acting in concert with Fgfs to promote organ specification and outgrowth in zebrafish, and that while Fgf expression may be unaffected in *hdac1* mutant embryos, loss of Hdac1 prevents mediation of Fgf signalling from occuring.

Previous studies have shown that Prox1 is required for organ bud formation, as Prox1 null mice display defects in migration of the hepatoblasts into the surrounding mesenchyme (Sosa-Pineda et al., 2000). Although the process of liver bud formation in zebrafish is still relatively unclear, the Prox1 positive hepatoblasts positioned in the ventral foregut at 24 hpf are thought to subsequently migrate anteriorly to form the liver bud. Therefore it is possible that lower levels of Prox1 in *hdac1* mutants results in an impaired ability of these cells to migrate, resulting in the defects observed in hepatic bud formation. In addition, Prox1 may control the degradation of ECM components surrounding the liver (Sosa-Pineda et al., 2000); this coupled with studies implicating Hdacs in the regulation of ECM components (Chiba et al., 2004) suggest that in *hdac1* mutants loss of deacetylase activity coupled with reduction of Prox1 expression may result in upregulation of ECM components inhibiting hepatoblast migration.

I used mosaic analyses to demonstrate that Hdac1 in the endoderm is required cell-autonomously for hepatic development. This is in line with findings demonstrating that isoforms of the intrinsic hepatic differentiation factor HNF4α interact with Hdac complexes to mediate hepatocyte maturation *in vitro* (Torres-Padilla et al., 2002) and studies in adult mice demonstrating Hdac1-mediated repression of C/EBPα expression through interaction with C/EBPβ in hepatocytes (Wang et al., 2008b) to promote hepatic proliferation. It is therefore possible that Hdac1 is acting to promote

competence in the endoderm, allowing the cells to respond to threshold levels of inductive signals from the overlying mesoderm. Interestingly, the latter study, in addition to work in mouse showing that Hdac1 interacts with p53 to repress expression of alpha-feto protein (Nguyen et al., 2005) suggests that Hdac1 interacts with components in the endoderm either promoting or repressing hepatic competence in a stage-specific manner. This highlights a possible mechanism whereby Hdac1 promotes expression of early hepatic genes during embryogenesis, but is required to silence them in the differentiated liver.

Analysis of mice and zebrafish depleted of *hdac1* revealed requirements for Hdac1 function in promoting cell proliferation (Cunliffe, 2004; Lagger et al., 2002; Stadler et al., 2005; Yamaguchi et al., 2005). My data demonstrated that liver hypoplasia in *hdac1* mutants results at least in part from decreased levels of proliferation. There are several candidate genes expressed in the liver that may mediate Hdac1-dependent proliferation. Hhex and Prox1 are both required to promote proliferation of the organ-forming endoderm, (Bort et al., 2004; Dyer et al., 2003; Kamiya et al., 2008; Sosa-Pineda et al., 2000). Due to the reduction of *hhex* and Prox1 expression in *hdac1* mutants at early stages of liver formation, this may suggest a mechanism by which levels of proliferation are reduced in *hdac1* mutant embryos.

Moreover, my data indicate that Hdac1 regulates cell proliferation through the early hepatic expression of the BRCT-domain containing factor Pes. In zebrafish *pes* mutants, the liver, pancreas and gut fail to expand (Allende et al., 1996) and cell culture studies have suggested a role for *pes* in cell cycle progression (Lerch-Gaggl et al., 2002). Analysis of liver size in *hdac1* mutants and embryos treated with TSA at later stages reveals that liver size remains significantly reduced at 72 and 96 hpf. At

these stages, levels of *pes* expression in *hdac1* mutants appear comparable with wild type, suggesting that other factors are involved in promoting proliferation and growth of the liver at these later stages, or that Hdac1 interacts with Pes to promote cell cycle progression. The expression of additional cell cycle genes were examined but were either not expressed in the liver at the stages examined, or no apparent differences were observed in levels of hepatic expression between wild type and *hdac1* mutant embryos. This may be due to insufficient sensitivity of the experimental techniques used, therefore analysis of protein levels may be more informative.

Downregulation of Hdacs, in particular Hdac1, has been shown to result in an upregulation of the cell cycle inhibitor p21 (Archer et al., 2001; Huang et al., 2005; Lagger et al., 2002; Ma and Schultz, 2008; Wang et al., 2005; Wilson et al., 2006). p21 expression in the developing digestive tract and its associated organs has been demonstrated in zebrafish (Chen et al., 2005). Therefore it is possible that loss of *hdac1* may result in upregulation of p21 in the liver, and subsequent downregulation of proliferation. Levels of hepatic p21 expression in *hdac1* mutants should be analysed to determine whether upregulation of this gene at later stages of liver outgrowth in *hdac1* mutants contributes to the reduction in liver size.

Further, it is interesting to speculate that Hdac1 is responsible for the regulation of genes involved in the length of the cell cycle. One hypothesis is that hepatocytes only differentiate after hepatoblasts undergo a certain number of cell cycles, similar to mechanisms suggested for Prospero-dependent neuronal differentiation in Drosophila (Choksi et al., 2006; Maurange et al., 2008). It is possible that in *hdac1* mutants, cell cycle length is increased, therefore the time taken for the number of cell cycles appropriate for hepatocyte differentiation is increased in these mutants, resulting in the observed proliferative defect and delay in differentiation. Alternatively, threshold

levels of genes expressed in the hepatoblasts may be required for this differentiation. Prox1 has been shown to be required for exit from the cell cycle, and proper differentiation of certain neuronal cell types in neural tissues and the retina in mouse (Dyer et al., 2003; Misra et al., 2008). Therefore it is possible that subtly reduced levels of Prox1 protein in the liver of *hdac1* mutant embryos are responsible for the defect in proliferation and the delayed onset of differentiation, as cells remain in a progenitor state.

Hdac1 in biliary development – mode of action

In *hdac1* mutants I observe a failure in intrahepatic ductal network refinement. However, it is unclear whether the defects in structure formation are the result of increased numbers and subsequent disorganisation of the cells comprising this system, or whether the defects are the result of misexpression of genes required for the refinement of this system. Members of the Notch signalling pathway as well as the Hnf family have been implicated in intrahepatic biliary system formation in mouse and zebrafish (Clotman et al., 2002; Margagliotti et al., 2007; Matthews et al., 2008; Matthews et al., 2005). Importantly, previous work in zebrafish has shown upregulation of components of the Notch pathway in the absence of *hdac1* activity (Cunliffe, 2004; Yamaguchi et al., 2005). Work mouse and zebrafish in addition to *in vitro* studies has suggested that upregulation of Notch signalling is important for directing hepatoblasts towards an intrahepatic duct fate (Ader et al., 2006; Lorent et al., 2004; Tanimizu and Miyajima, 2004); Notch signalling is activated in hepatoblasts which undergo differentiation into cholangiocytes in mouse. This suggests an interesting role for *hdac1* in biliary development, possibly acting via upregulation of the Notch signalling pathway, or interacting with members of the *hnf*

family in segregating and specifying biliary cell lineage from bipotent hepatoblasts in the formation of the intrahepatic biliary system.

Recently the timely formation of the biliary system in zebrafish has been shown to be tightly linked with the invasion of endothelial cells and subsequent vascularisation of the liver (Sakaguchi et al., 2008). It has been demonstrated that both Hdac1 and Hdac3 are required for vascularisation of the liver (Farooq et al., 2008), and it is possible that this defect in angiogenesis is partly or entirely responsible for the delay in formation of the intrahepatic biliary system in *hdac1* mutants.

Additionally, in *hdac1* mutants, structural differentiation of the extrahepatopancreatic ducts is affected, and morphology appears perturbed. It is interesting to speculate that the observed expansion of early extrahepatopancreatic ductal tissue is also due to an upregulation of the Notch signalling pathway in this part of the organ-forming endoderm, which is required to maintain extrahepatopancreatic ductal system fate (Sumazaki et al., 2004).

Roles for Class I histone deacetylases in liver organogenesis

The use of TSA results in a more severe hepatic phenotype than observed in *hdac1* mutants, suggesting that additional Hdacs are required in hepatic development. I utilised morpholinos against *hdac 3*, *8*, and *11* to investigate potential roles for these genes in liver formation.

Early liver formation appears to progress independent of Hdac8 and 11 function. While Hdac11 may be required for growth and differentiation of the liver at later stages of endodermal organogenesis, I did not observe *hdac11* expression in the liver at later time points. However, as TSA treatment suggested a requirement for Hdacs

during somitogenesis stages for hepatic differentiation, it is possibly that *hdac11* is acting at this time to drive hepatic differentiation.

I observed a small reduction in liver size in *hdac3* morphants at 4 dpf, and more severe defects in hepatic differentiation and growth in embryos depleted of Hdac1 and Hdac3 function, in agreement with a reported role for Hdac3 in hepatic growth, and potentially a combined requirement for *hdac3* and *hdac1* in hepatic differentiation (Farooq et al., 2008). Interestingly, I was unable to reproduce the early hepatic specification and differentiation phenotypes described in *hdac3* morphants (Farooq et al., 2008). This discrepancy may be due to slightly different morpholinos used to target the transcriptional start site in both studies, suggesting different efficacies of morpholino. However, this possibility seems unlikely as consistent defects were observed in the hindbrain upon examination of *ptf1a* expression at 48 hpf (data not shown), suggesting that functional knockdown was effective at the dosage injected. Nevertheless, I cannot rule out the possibility that the injected concentrations of morpholino were not sufficient to induce an early hepatic phenotype. Importantly, *hdac3* is expressed in the liver at 48 hpf and beyond supporting the data presented here, in addition to studies by Farooq and colleagues, that both genes are required for liver differentiation and growth.

Strikingly, liver specific knockdown of Hdac3 in post-partum mice results in liver hypertrophy as the result of increased levels of cell proliferation (Knutson et al., 2008). This work may highlight the different roles for Hdacs during embryogenesis and adult processes, or potentially raise the possibility of organism specific requirements for Hdacs. Work in mouse has shown different requirements for Hdac1 in young and old mice, whereby Hdac1 promotes liver proliferation in young mice, but inhibits liver proliferation in old mice (Wang et al., 2008a; Wang et al., 2008b),

thereby supporting the possibility that not only Hdac1, but additional Hdacs could play opposing roles in the same organ at different stages of an organisms life.

Hdac1 functions in pancreatic organogenesis

Hdac1 is a widely expressed gene, therefore I examined pancreatic development in *hdac1* mutants, and found partially similar and very different requirements for Hdac1 in pancreas formation. My data demonstrate that Hdac1 is required for morphogenesis of the endocrine islet, in addition to timely specification and differentiation of the exocrine pancreas. The molecular events surrounding endocrine islet formation are beginning to emerge. Endocrine cells are initially specified as bilateral groups of cells that are positioned along the anteroposterior axis which subsequently aggregate into a single islet (Argenton et al., 1999; Biemar et al., 2001; Huang et al., 2001). Interestingly, *hdac1* mutants display defects in the positioning of the endocrine pancreas, with ectopic anterior groups of cells present from 24 hpf onwards. Here, I have shown a mild delay in endocrine cell specification in *hdac1* mutants in addition to impaired migration of these cells. Alternatively, the mild delay in endocrine specification may cause cells specified later arising in more anterior positions, which subsequently fail to migrate posteriorly. It may be possible that the timely specification of endocrine cells is crucial for their ability to migrate posteriorly. This finding suggests that Hdac1 is required for islet aggregation, either within islet cells or in neighbouring tissues promoting this process. Alternatively, Hdac1 may repress ectopic endocrine cell formation in endodermal tissue anterior to the main islet, however, I cannot currently distinguish between these possibilities. To determine precisely the early defect observed in *hdac1* mutants, it would be necessary to view islet formation using a transgenic line expressing GFP under the control of the

insulin promoter (Huang et al., 2001), allowing real time analysis of the temporal and spatial induction and subsequent aggregation of endocrine cells in *hdac1* mutant embryos.

Endocrine cell specification and positioning is regulated through factors including Retinoic acid (RA) and *sonic hedgehog.* Increased RA signalling, or inhibition of Hh signalling (diIorio et al., 2002; Stafford and Prince, 2002), gives rise to a phenotype comparable to that observed in *hdac1* mutants, with the latter appearing qualitatively more similar to the *hdac1* mutant phenotype. Preliminary analysis revealed no defects at 24 hpf in the domain of *shh* expression in *hdac1* mutant embryos (data not shown), however it will be necessary to analyse *shh* expression at mid-somitogenesis stages, when it is required for endocrine specification (Chung and Stainier, 2008). . In the zebrafish hindbrain, Hdac1 is required in neural precursor cells to maintain responsiveness to Hh signalling (Cunliffe, 2004). Therefore, it is possible that interactions between Hdac1 and Hh signalling are responsible for endocrine islet formation, rather than Hdac1 regulating the expression of *shh*.

RA produced in the mesoderm is required to signal to RA receptors in the adjacent endoderm to specify *insulin* producing cells (Stafford et al., 2006). In addition to this requirement in endocrine pancreas establishment, RA plays a role in the positioning of these cells with respect to the anteroposterior axis. It is possible that in *hdac1* mutants a defect in RA signalling leads to aberrant specification of *insulin* expressing cells in more anterior positions.

Recent work in chick and mouse, as well as zebrafish has implicated complex interactions between the pancreatic endoderm and the adjacent LPM for formation of the pancreatic anlage, and in zebrafish in particular for exocrine pancreas specification and outgrowth (Cano et al., 2007; Manfroid et al., 2007).

Fgf signalling from the LPM has been implicated in exocrine pancreas formation (Cano et al., 2007; Manfroid et al., 2007). In zebrafish, loss of Fgf24 function leads to reduced or absent *meis3* and *islet1/2* expression in the neighbouring LPM and a reduced exocrine pancreas anlage, while loss of both Fgf24 and Fgf10 together results in severely reduced or absent exocrine pancreas highlighting redundant requirements for these two Fgfs in exocrine pancreas formation (Manfroid et al., 2007). A requirement for Hdac activity in mediating Fgf signalling has been shown in *X.laevis* mesoderm induction and zebrafish development (Plaster et al., 2007; Xu et al., 2000). It will thus be interesting to examine additional potential interactions between Fgf signalling and Hdac1 function with regards to the establishment of the exocrine pancreas. Importantly, the unaltered levels of expression of the TALE-box protein *meis3* in the LPM, which is regulated by Fgf signalling in the endoderm, and is required for formation of both pancreatic anlage, suggest that Hdac1 is not mediating expression of, or interacting with, Fgfs in patterning the pancreatic LPM. I cannot rule out that interactions between Hdac1 and Meis3 are required for initiation of both pancreatic programmes, and previous studies have shown that Hdacs interact with Meis proteins and their binding partners (Saleh et al., 2000). In addition, preliminary data suggest *meis1* is expressed in the exocrine pancreas after organ specification occurs (data not shown), therefore Hdac1 could interact with Meis1 to promote differentiation of this organ.

While *meis3* expression levels in the LPM appear unaltered in *hdac1* mutants, the LPM is misplaced at 30 hpf. Work in zebrafish suggests that different domains of the LPM express different inductive signals, which may then signal to separate regions of the underlying endoderm (Chung and Stainier, 2008; Huang et al., 2008; Manfroid et al., 2007). It is possible that in *hdac1* mutants, the mispositioning of the LPM results

in improper placement of inductive signals in relation to the abutting organ-forming endoderm, leading to the specification defects observed.

Strikingly, in the majority of *hdac1* mutants, analysis of exocrine pancreas specification at 72 hpf revealed positioning of these cells on the left side of the embryo, in contrast to the positioning of the exocrine pancreas on the right side of the embryo at this time. In wild type embryos the exocrine pancreas initiates posterior to the liver on the left side of the midline, before projecting to the right side of the digestive tract. Therefore, it is possible that the left side positioning of exocrine cells in *hdac1* mutants at 72 hpf reflects the fact that these cells are newly specified posterior to the liver as in wild type embryos, albeit with a delay, and are not mispositioned. Indeed, analysis of exocrine pancreas formation at 4 dpf in *hdac1* mutants utilising transgenic lines expressing GFP in the pancreas under the control of the *elastaseA* promoter revealed that despite defects in outgrowth, this organ anlage is not mispositioned at this time. To resolve this point, analysis of greater numbers of embryos may be combined with analysis of the positioning of the newly specified exocrine pancreas cells in *hdac1* mutants by in situ hybridisation in conjunction with genes expressed in the liver, to confirm the positioning of these two cell populations in relation to each other.

The fact that pancreas specification occurs with a delay of at least 30-40 hours suggests that the endoderm either maintains or gains the competence to respond to delayed exocrine pancreas specification signals in *hdac1* mutants. This is reminiscent of recent findings in hepatic development, where transient inhibition of Fgf and Bmp signalling in zebrafish delayed hepatoblast formation and differentiation for about 16 hours, suggesting that the endoderm maintains its competence to respond to specification and differentiation signals (Shin et al., 2007).

Tissue specific requirements for Hdac1

hdac1 mutants display defects in both endodermal and adjacent mesodermal tissues. This raises the possibility that Hdac1 is directing liver formation through the regulation of activating and repressing factors within the mesoderm, or that Hdac1 is acting within the endoderm to promote hepatic organogenesis. In support of the first possiblity, reduced *prt/wnt2bb* expression is observed in the LPM of *hdac1* mutants. However, due to the nature of Hdac1 as a traditional transcriptional repressor, it is unlikely that this is a direct interaction. Alternatively, as I have demonstrated a cell-autonomous role for Hdac1 within the endoderm for hepatic development, it is possible that Hdac1 promotes signalling to the overlying mesoderm to induce factors such as *prt/wnt2bb* required for hepatic specification, similar to the role of Fgf24 in exocrine pancreas specification (Manfroid et al., 2007). However, if this was the only requirement for Hdac1, mosaic analysis should have revealed cells expressing *cp* other than solely those wild type cells transplanted to the endoderm of *hdac1* mutants. In turn, this supports the hypothesis that *hdac1* is acting within the endoderm to promote hepatic competence, allowing these cells to respond to signals from the overlying mesoderm. However, I cannot rule out that these processes occur in parallel, or that *hdac1* is required in different roles in both tissues to promote liver specification, differentiation and growth. In order to further investigate this possibility it will be interesting to replace the endoderm in *hdac1* mutants with wild type endoderm, and assess the impact of the endoderm on expression of mesodermally derived hepatic induction factors, such as *prt/wnt2bb*.

Hdac1 ensures correct cell allocation to different organ fates in zebrafish

In the absence of Hdac1 function, I observed an increase in the number of non-hepatic foregut cells forming at the expense of the liver and exocrine pancreas. This results in an increased contribution of non-hepatic foregut tissue to the total endoderm in the organ-forming region, suggesting that Hdac1 is required to promote liver and pancreas specification from the foregut endoderm. It is unclear whether the role for Hdac1 in decisions between hepatic, pancreatic and foregut tissue is instructive or permissive, and whether in the absence of hepatoblast specification signals, the gut tissue differentiates into a digestive tract fate. Work in mouse has suggested that in the absence of liver induction, foregut endodermal cells give rise to pancreatic cells (Deutsch et al., 2001), suggesting a default pancreatic fate in the absence of hepatic instructive signals, however, studies in zebrafish in addition to the data presented here, does not support this mechanism in zebrafish (Chung et al., 2008).

In zebrafish, endodermal organogenesis occurs as a series of steps. Initially, the endoderm is specified and subsequently regionalised giving rise to anterior organ-forming endoderm and posterior intestine. The anterior endoderm then acquires a foregut fate, and subsequently gains the competence to become liver or pancreas. My data suggest that Hdac1 is acting in the last step of organogenesis, allowing a subset of foregut cells to become either liver or pancreas.

Interestingly, despite an increase of foregut tissue, I fail to observe an increase in gut differentiation. Conversely, *hdac1* mutants exhibit a mild reduction in *vhnf1* expression, followed by a slight delay in the onset of *claudin15*, both genes required for digestive tract lumen formation (Bagnat et al., 2007). Knockdown of RA signalling results in a loss of foregut differentiation (Nadauld et al., 2005) similar to that observed in *hdac1* mutants, again raising the possibility that Hdac1 mediates its effects upon the endoderm through RA signalling. However, the ectopic endocrine

pancreas phenotype in *hdac1* mutants is similar to that observed upon increased RA signalling, as previously discussed (Stafford et al., 2006). Therefore it is possible that either Hdac1 differently regulates RA signalling in different stages of endodermal organogenesis, or that RA is not the common signalling pathway by which Hdac1 is mediating its effects upon the endoderm. Application of RA to *hdac1* mutant embryos, and subsequent analysis of both endocrine pancreas and intestinal differentiation may help determine whether *hdac1* is acting through RA in promoting the formation and differentiation of these tissues.

In addition to the increase in foregut tissue in *hdac1* mutants, I observe a concomitant defect in lumen formation. Lumen formation in zebrafish proceeds by the formation of several foci, a process which is thought to occur through fluid accumulation, and subsequent tissue rearrangement results in the formation of a single lumen. It seems possible that the tissue rearrangements required for the final stages of single lumen formation are disrupted due to the increased number of cells found in the foregut endoderm. It has also been suggested that ECM components are required for this final step of lumen formation (Bagnat et al., 2007). In line with this, Hdac1 has been shown to regulate the expression of ECM components (Chiba et al., 2004; Whetstine et al., 2005), and preliminary data suggest an increase of Laminin expression in *hdac1* mutants. Therefore, it will be important to examine further the expression of other ECM components such as Fibronectin and Collagen in order to determine whether aberrant expression of these proteins may account for lumen formation defects observed in *hdac1* mutants. Together, *hdac1* mutants may represent a good model to examine the consequences of increased foregut cell numbers and altered levels of ECM components on gut formation.

Hdacs mediate chromatin compaction, resulting in transcriptional repression. Strikingly, I have not identified targets of Hdac1 which are upregulated in the organ-forming region in *hdac1* mutants. It has been demonstrated that *hdac3* regulates liver development through repression of *gdf11* (Farooq et al., 2008). Work in mouse has highlighted a role for *gdf11* for formation of the endocrine pancreas (Harmon et al., 2004), where knockdown of GDF11 results in increased endocrine progenitors. In addition, loss of *gdf11* in zebrafish results in an expansion of the exocrine pancreas (Farooq et al., 2008). It is therefore interesting to speculate that Hdac1 may also be acting through regulation of *gdf11* in formation of only the liver and both pancreatic lineages.

I have demonstrated that *hdac1* plays multiple yet distinct roles in liver and pancreas organogenesis. Further analysis will be required to determine targets by which *hdac1* mediates the progression of these processes. Microarray analysis of hepatic and non-hepatic endoderm in wild type embryos and *hdac1* mutants will aid identification of Hdac1 targets, and subsequent functional analysis of Hdac1 target genes will elucidate the tissue specific mechanisms of Hdac1 activiy in endodermal organogenesis.

Chapter 4

4C1 is required for endodermal organogenesis in zebrafish

4.1 4C1 is required for hepatic and pancreatic development

The mutant line *4C1* was also identified as part of the forward genetic screen to identify mutants with defects in endodermal organogenesis. *4C1* mutants exhibits liver hypoplasia and a dysmorphic exocrine pancreas at 48 hpf (Fig. 4.1, E, F) and can be identified from around 26 hpf onwards by defects in body elongation, a blistered tail, pigmentation defects, and heart oedema (Fig. 4.1 A-D).

To characterise hepatic development in the context of the forming digestive system in *4C1* mutant embryos, I analysed early stages of liver formation using the *Tg(gutGFP)*^{s854} line in conjunction with α-Prox1 and α-Islet 1/2 staining. At 24 hpf in wild type embryos, the endoderm represents a multicellular rod at the embryonic midline in which newly specified Prox1-expressing hepatoblasts reside ventrally in the presumptive foregut region (Fig. 4.2, A). In *4C1* mutant embryos at the same stage Prox1-positive cells are detected however their number appears reduced (Fig. 4.2, H). As liver bud formation progresses in wild type embryos, the Prox1-positive hepatoblasts aggregate anteriorly on the left side of the organ-forming region to form a liver bud by 30 hpf (Fig. 4.2, B, C). In *4C1* mutant embryos, Prox1-positive cells fail to aggregate in a more anterior position until around 36 hpf (Fig. 4.2, I-K). In addition, leftward looping of the gut and leftward outgrowth of the liver bud fail to occur up to this stage. Although a liver bud forms in *4C1* mutant embryos, it is significantly reduced in size when compared to wild type embryos at 48 hpf (Fig. 4.2, compare G, N).

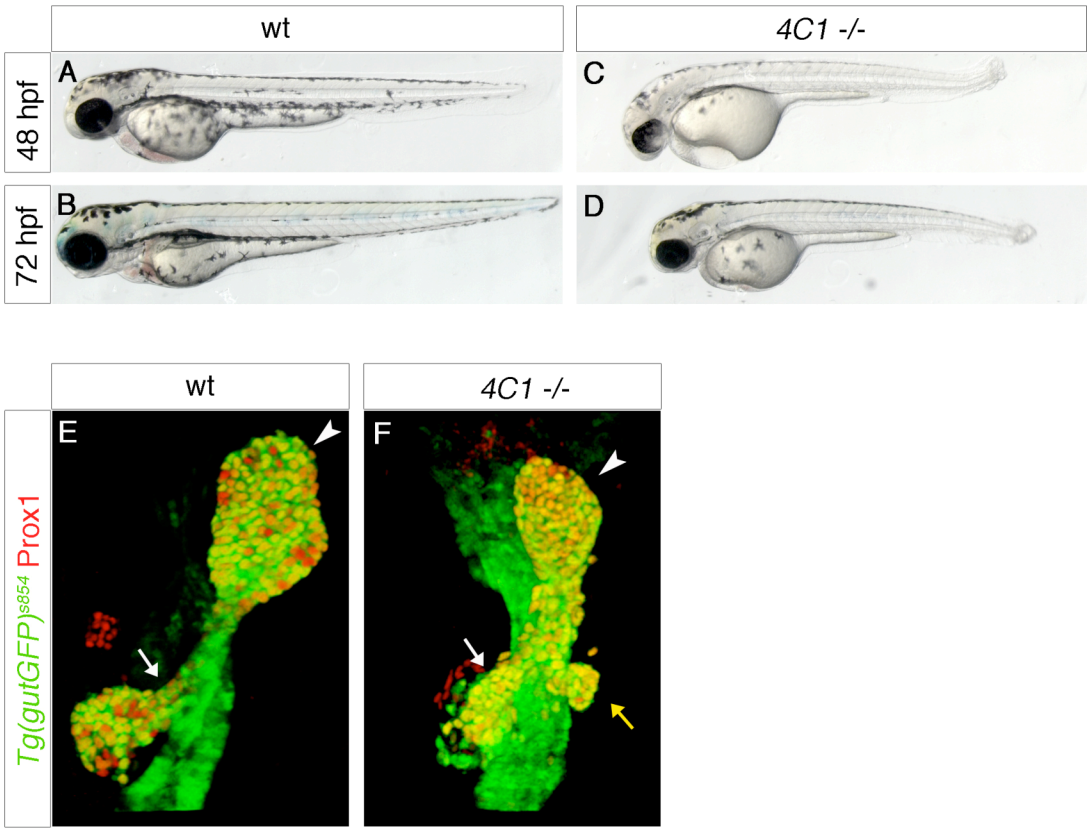


Figure 4.1 4C1 is required for hepatic and pancreatic organogenesis in zebrafish

(A-D) Brightfield pictures of wild type (A, B) and *4C1* mutant embryos (C, D) at 48 and 72 hpf. (E, F) Ventral projection of confocal stacks of wild type (E) and *4C1* mutant embryos (F) at 48 hpf. *Tg(gutGFP)*^{s854} is expressed throughout the endoderm (green), and embryos were stained for Prox1 (red) highlighting the cells of the liver (arrowhead) and exocrine pancreas (white arrow; yellow arrow indicates ectopic bud).

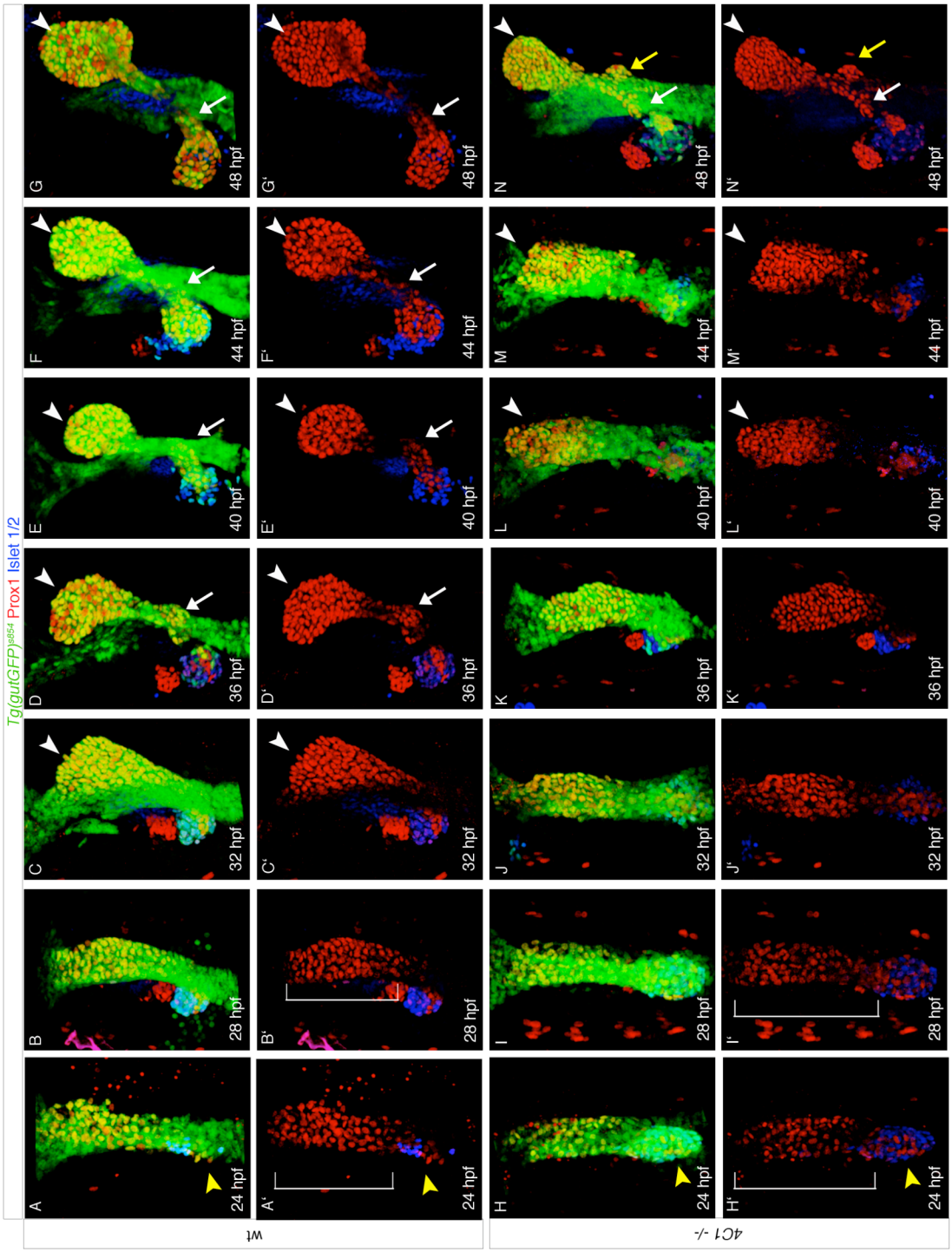

Tg(gutGFP)s854 Prox1 Islet 1/2
wt
4C1 -/-
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'
I
I'
J
J'
K
K'
L
L'
M
M'
N
N'
24 hpf
28 hpf
32 hpf
36 hpf
40 hpf
44 hpf
48 hpf

Figure 4.2 4C1 plays multiple roles in liver and pancreas organogenesis.

Time-course analysis of endodermal organogenesis in wild type and *4C1* mutant embryos using the *Tg(gutGFP)*^{s543} transgene to highlight the endoderm (green). Prox1 (red) is expressed in hepatoblasts and the exocrine pancreas, and Islet 1/2 expression marks the endocrine pancreas. Ventral projections of confocal stacks, anterior to top. (A-G'), wild type embryos, (H-N'), *4C1* mutant embryos. (A, A') At 24 hpf, newly specified Prox1-positive hepatoblasts are found in the ventral foregut domain; at 28 hpf they begin to aggregate anteriorly on the left side of the embryo (B, B'), such that by 32 hpf the liver bud has formed (arrowhead, C, C'). In *4C1* mutants, a reduced number of Prox1-positive hepatoblasts form in the ventral foregut domain (bracket, H,H'), which fail to aggregate in a more anterior position until around 36 hpf (K, K'). The liver bud subsequently grows out to the left side of the digestive tract (arrowhead, L, L'), resulting in a hypoplastic liver bud at 48 hpf (arrowhead, N, N'). In wild type embryos the exocrine pancreas is initiated as a Prox1-positve group of cells posterior to liver at 34 hpf (arrow, D), which project ot the right side of the endoderm, engulfing the endocrine islet by 48 hpf (arrow, G). The exocrine pancreas fails to be initiated in *4C1* mutants at 34 hpf, however Prox1-positive cells are observed posterior to the liver at 44 hpf (M). By 48 hpf a population of exocrine cells project to the right side of the digestive tract (white arrow, N), in addition to an ectopic Prox1-positive bud posterior to the liver (yellow arrow, N). White arrowhead, liver; white arrow, exocrine pancreas; yellow arrowhead, endocrine pancreas.

In wild type embryos, the neighbouring exocrine pancreas is specified around 34 hpf, as a group of Prox1-positive cells posterior to the liver bud (Fig. 4.2, D). These cells subsequently grow out to the right side of the embryo, engulfing the endocrine pancreas and ultimately connect a single pancreas to the adjacent alimentary canal by the common extrahepatopancreatic duct (Fig. 4.2, G). In *4C1* mutants, Prox1-positive cells posterior to the hepatic region are first observed around 44 hpf (Fig. 4.2, M), and by 48 hpf the exocrine pancreas anlage projects to the right side of the digestive tract (Fig. 4.2, N). In the majority of *4C1* mutants, an additional Prox1-positive bud is situated posterior to the liver, and appears to be separate from the main exocrine anlage (Fig. 4.2, N). As both liver and exocrine pancreas express Prox1 at this stage, it is possible that this ectopic anlage could be either hepatic or pancreatic. In addition, the extrahepatic duct which is Prox1-negative in wild type embryos does not appear to have formed in *4C1* mutants at this stage, and Prox1 is ectopically expressed in the presumptive duct region. Interestingly, formation of the Islet1/2-positive endocrine pancreas appears unaffected in *4C1* mutants (Fig. 4.2, A, H). Together, these data suggest a role for *4C1* in formation of the liver as well as the extrahepatopancreatic ductal system, and the exocrine pancreas.

4.2 4C1 is required for specification of correct hepatoblast number and liver morphogenesis

Analysis of hepatoblast specification in 4C1 mutants by Prox1 expression at 24 hpf, suggests that 4C1 is not required for timely initiation of hepatoblast specification but is instead required for specification of the correct number of hepatoblasts. In order to further investigate the requirement for *4C1* in hepatoblast specification, I examined

hhex expression, which along with Prox1 is one of the earliest genes expressed in newly specified hepatoblasts from 22 hpf onwards (Fig. 4.3, A, B; Field et al., 2003b). Similar to Prox1 expression, *4C1* mutant embryos express *hhex* at 24 hpf in the newly specified hepatoblasts and expression is maintained in the liver at 48 hpf. Nevertheless, the expression domain appears reduced at both stages (Fig. 4.3, C, D), in agreement with the observed reduction in Prox1-positive hepatoblast number, confirming that *4C1* is required for formation of the correct number of hepatoblasts. In order to understand further the hepatic phenotype in *4C1* mutants, I determined whether hepatoblast differentiation is initiated in *4C1* mutants. Hepatocyte differentiation begins at around 34 hpf and is primarily marked by the expression of genes such as the plasma protein *cp* (Fig. 4.3, E, F). Analysis of *cp* expression in *4C1* mutants reveals that hepatic differentiation occurs in the majority of *4C1* mutants by 48 hpf (95%, n= 20, Fig. 4.3, G), although levels of *cp* expression appear slightly reduced, and expression is present in 100% of embryos at 72 hpf (Fig. 4.3, H). This suggests that in the absence of *4C1*, a slight delay in liver differentiation may occur, although detailed analysis of *cp* expression between 34 and 48 hpf would be required to further highlight a potential requirement for *4C1* in hepatic differentiation. Furthermore, *cp* expression highlights the reduced size and abnormal morphology of the liver at both 48 and 72 hpf, suggesting that *4C1* plays an important role in the morphogenesis and growth of this organ.

Analysis of the organ-forming region using the *Tg(gutGFP)*^{s854} line revealed that the Prox1-positive hepatoblasts appear to remain in a more posterior position in the organ-forming region in *4C1* mutants, suggesting defects in the positioning or migration of these cells in liver bud formation. Using Volocity imaging software, I manually marked the anterior tip of the endocrine islet, and the posterior-most Prox1-

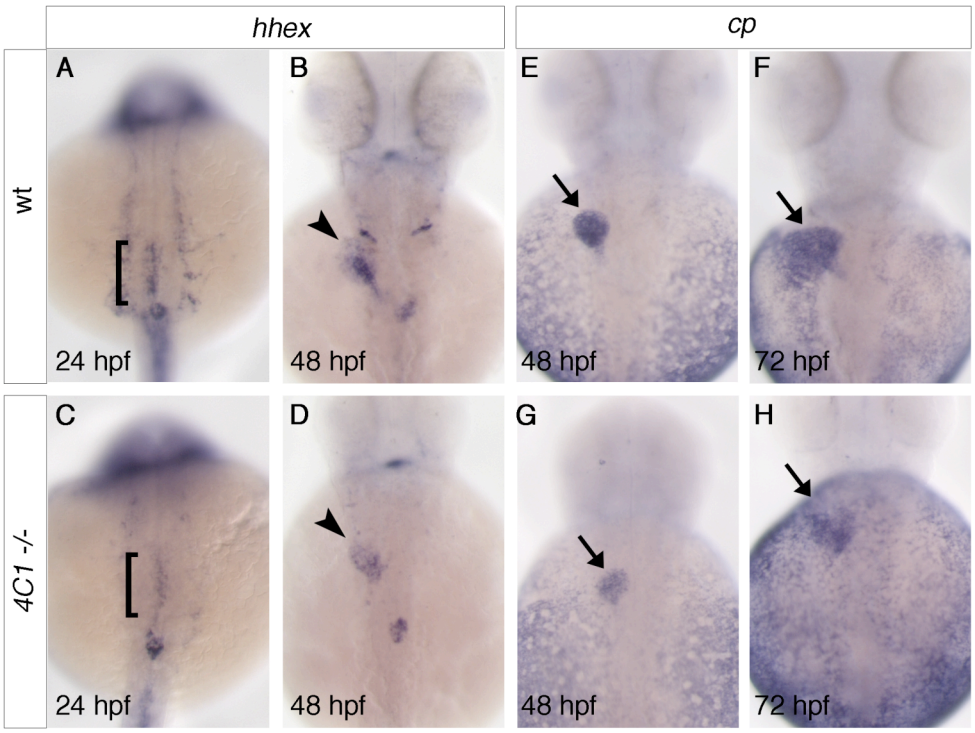
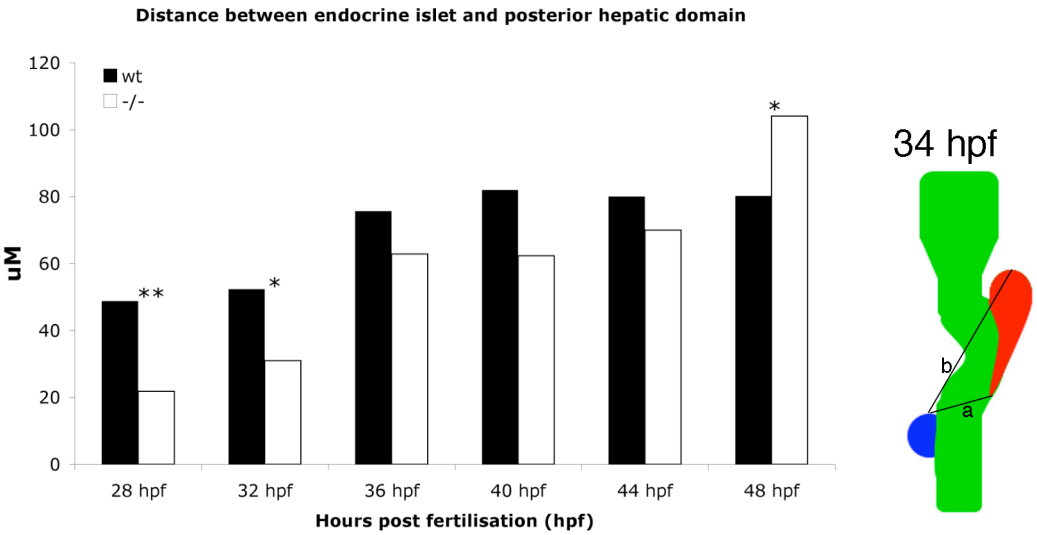


Figure 4.3 4C1 is dispensable for timely liver specification and onset of differentiation.

In situ hybridisation analyses of *hhex* and *cp* expression in *4C1* mutants, dorsal views, anterior to top. Wild type embryos express *hhex* in forming hepatoblasts at 24 hpf (bracket, A), and in the liver at 48 hpf (arrowhead, B). *4C1* mutant embryos exhibit *hhex* expression at both 24 hpf (bracket, C), and 48 hpf (arrowhead, D) in a reduced number of cells. Wild type embryos express *cp* in the liver from around 34 hpf onwards (arrow, E, F), and *4C1* mutants express *cp* at 48 and 72 hpf (arrow, G, H) in a reduced domain.

A



B

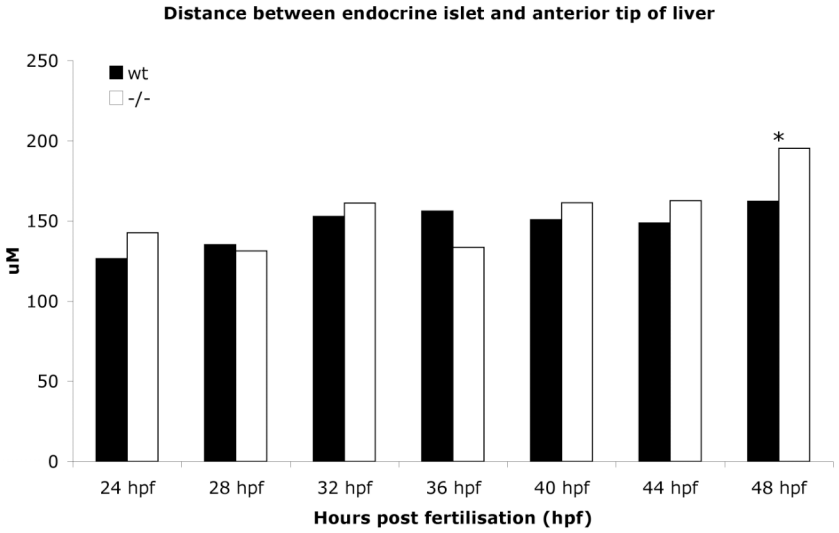


Figure 4.4 4C1 mutants display defects in hepatoblast positioning

(A) Distance between the anterior tip of the endocrine islet and the posterior end of the hepatic domain in wild type and *4C1* mutant embryos. The hepatic domain extends more posteriorly in *4C1* mutants when compared to wild type embryos at 28 and 32 hpf, however, by 48 hpf, the liver is found more anteriorly than in wild type. (B) Distance between the anterior tip of the endocrine islet and the anterior tip of the hepatic domain in wild type and *4C1* mutant embryos. There is no significant anterior shift in the anterior domain of the hepatic region in *4C1* mutants when compared to wild type embryos until 48 hpf. Schematic depicts distances measured for data sets A and B.

positive hepatoblast in the liver and measured the distance between these two anlagen in wild type and *4C1* mutants, in addition to the overall size of the organ-forming domain. Initially, the distance between the endocrine pancreas and the hepatic domain is significantly smaller at 28 hpf, and remains smaller at 32 hpf (Fig. 4.4, A). However, after this time the distance remains slightly smaller but not significantly so, until 48 hpf when the liver in *4C1* mutant embryos appears more anterior than in wild type embryos (Fig. 4.4, B). Interestingly, quantification of the distance between the endocrine islet and the anterior tip of the liver domain between 28 and 48 hpf in wild type and *4C1* mutants revealed no defects (Fig. 4.4, B) suggesting that at early stages, the size of the Prox1-positive hepatic domain is expanded posteriorly in *4C1* mutants.

Altogether, these results show that *4C1* is required for controlling the number and positioning of hepatoblasts and subsequent morphogenesis of the developing liver.

4.3 4C1 is required for biliary system morphogenesis

The formation of the biliary system, connecting the liver and pancreas via a common extrahepatopancreatic duct to the adjacent digestive tract, is tightly connected to the formation of a functioning liver and pancreas. Analysis of liver and pancreas organogenesis at 48 hpf using the *Tg(gutGFP)*^{s854} line revealed that duct morphology is disrupted at this stage (Fig. 4.2) To assess the requirement for *4C1* in biliary system formation, the expression of 2F11 was analysed. 2F11 is expressed from around 40 hpf onwards in wild type embryos (Fig. 4.5, A) in the liver, endocrine pancreas and presumptive ductal tissue between the liver and pancreas, and from 48 hpf onwards becomes downregulated in the liver and heightened in the intra- and extrahepatic ducts (Fig. 4.5, B-D). Analysis of 2F11 expression reveals that *4C1*

mutant embryos initiate 2F11 expression similar to wild type embryos at 40 hpf (Fig. 4.5, E). However, by 48 hpf 2F11 expression fails to become downregulated in the liver (Fig. 4.5, F), and in the majority of *4C1* mutants at 72 hpf and 96 hpf, 2F11 expression in the liver remains uniform and refinement of the intrahepatic ducts appears perturbed with fewer branching ducts visible (Fig. 4.5, G, H). Interestingly, Prox1 expression which in wild type embryos is excluded from the extrahepatic duct from 48 hpf onwards (Fig. 4.5, B), is expanded into the 2F11 expressing domain at 48 hpf in *4C1* mutants and is not excluded from the extrahepatic duct until 72 hpf (Fig. 4.5, F). Additionally, from 72 hpf onwards the 2F11 expressing domain appears expanded in *4C1* mutants, likely reflecting an increase in ductal tissue in these embryos (Fig. 4.5, G, H). The expansion of the extrahepatic duct may be due to the presence of ectopic exocrine pancreatic buds, as the liver and both pancreatic anlage are all connected to the adjacent foregut by the ductal structures of the biliary network. Sections through the duct at this stage reveal that lumen formation in the duct appears to occur (data not shown), suggesting that despite an increase in duct structures, the individual ducts are undergoing correct lumen formation. Additionally, 2F11 is ectopically expressed at 72 and 96 hpf in the alimentary canal directly posterior to the forming extrahepatopancreatic duct (Fig. 4.5, G', H').

Together, these data suggest a role for 4C1 in segregation of the hepatocyte and intrahepatic biliary lineages, in addition to the morphogenesis and patterning of the extrahepatopancreatic ductal system.

4.4 4C1 is required for exocrine pancreas morphogenesis

Analysis of endodermal organogenesis in *4C1* mutants suggested that in addition to a hypoplastic liver, the exocrine pancreas was reduced in size and an ectopic organ

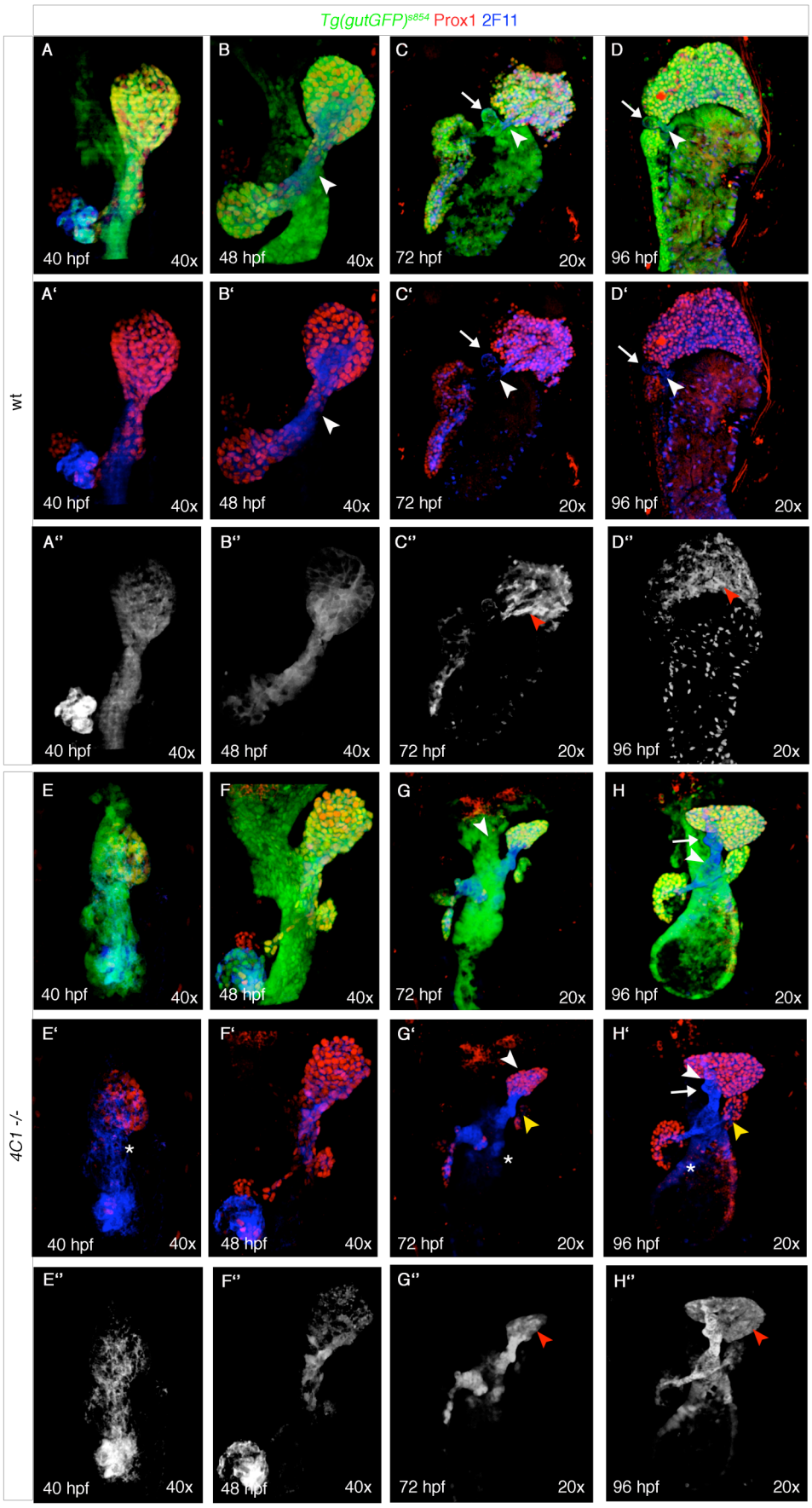

Tg(gutGFP)s854 Prox1 2F11
wt
4C1 -/-
A
B
C
D
A'
B'
C'
D'
A''
B''
C''
D''
E
F
G
H
E'
F'
G'
H'
E''
F''
G''
H''
40 hpf
48 hpf
72 hpf
96 hpf
40x
20x

Figure 4.5 4C1 is required for hepatopancreatic duct morphogenesis

Time-course analysis of biliary system formation in wild type (A-D'') and *4C1* (E-H'') mutant embryos, expressing *Tg(gutGFP)*^{s854} throughout the endoderm (green), and stained for Prox1 (red) highlighting the liver and exocrine pancreas, and 2F11 (blue) expressed in the ductal tissue and endocrine pancreas. Ventral projections of confocal stacks, anterior to top.

Both wild type embryos and *4C1* mutants express 2F11 in the liver, pancreas and presumptive ductal tissue between these two organs from around 40 hpf (A, E). From 48 hpf wild type embryos hepatic 2F11 expression is downregulated in hepatocytes and become heightened in the intrahepatic ducts within the liver (B, C), however in *4C1* mutants differentiation of defined intrahepatic ducts appears to fail (red arrowhead, G'', H''). Futhermore, in wild type embryos from 48 hpf expression of 2F11 is heightened in, and Prox1 expression is excluded from, the forming extrahepatopancreatic duct (arrowhead, B-D). In *4C1* mutants at 48 hpf, expression of 2F11 appears to be heightened in the duct, and Prox1 is ectopically expressed in the duct (F). From 72 hpf, 2F11 expression reveals a dysmorphic ductal system (white arrowhead, G, H); the amount of ductal system tissue appears increased and a third duct is present connecting the ectopic exocrine pancreas to the foregut (yellow arrowhead, G, H). I observe an expansion of 2F11 expression into the foregut directly posterior to the organ-forming region in *4C1* mutants at both 72 and 96 hpf (asterisk, G, H).

anlage was budding posterior to the liver (Fig. 4.2). Expression analysis of hepatic genes such as *hhex* and *cp* suggested that this is not an ectopic hepatic bud (Fig. 4.3), raising the possibility that *4C1* mutants develop an ectopic exocrine pancreas. To compare the role of *4C1* in hepatic development with a possible requirement in exocrine pancreas formation, I examined the expression of *ptf1a* and *trypsin*. *ptf1a* is expressed in the newly initiated exocrine pancreas from around 34 hpf onwards (Fig. 4.6, A, B; Lin et al., 2004). Analysis of *4C1* mutants at 48 hpf revealed that the *ptf1a*-positive exocrine pancreas is on the right side of the embryo, however the exocrine anlage is reduced in size compared to wild type embryos (Fig. 4.6, C). Additionally, a second group of exocrine cells is present on the left side of the embryonic midline, posterior to the developing liver bud (Fig. 4.6, C, D). *trypsin*, a marker of exocrine function, is expressed in the pancreas from 48 hpf onwards (Fig. 4.6, E, F). Similarly to *ptf1a* expression, analysis of *trypsin* expression in *4C1* mutants revealed a reduced exocrine bud on the right side of the digestive tract and a second exocrine pancreas on the left side (Fig. 4.6, G, H), suggesting that the ectopic Prox1-positive bud observed using the *Tg(gutGFP)*^{s854} *4C1* line represents an ectopic exocrine pancreatic bud. Altogether, exocrine pancreatic gene expression is initiated independent of 4C1 function, however it appears to be required for exocrine pancreatic cell number and integrity and outgrowth of the exocrine pancreas bud.

Initial characterisation of endodermal organogenesis in *4C1* mutants suggested that endocrine islet formation was unaffected. To confirm that *4C1* is dispensable in endocrine pancreas formation, I examined endocrine pancreas formation further in *4C1* mutants. Both wild type embryos and *4C1* mutants express *insulin* in the β-cells of the endocrine islet which forms a single cluster at the posterior end of the organ-

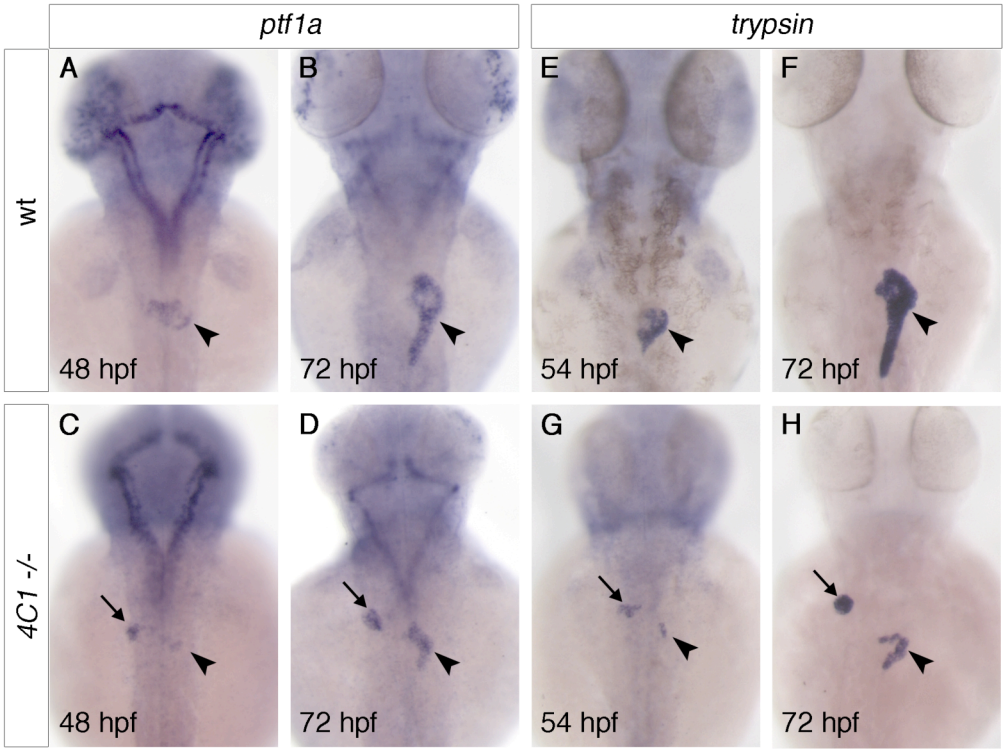


Figure 4.6 4C1 is required for exocrine pancreas morphogenesis

In situ hybridisation analyses of *ptf1a* and *trypsin* expression, dorsal views, anterior to top. Wild type embryos express *ptf1a* in the exocrine pancreas from around 34 hpf, onwards (arrowhead, A, B). *4C1* mutants express *ptf1a* in an overall reduced exocrine pancreas at 48 and 72 hpf (arrowhead, C, D). In addition, in *4C1* mutants an ectopic group of *ptf1a* expressing cells is present on the left side of the embryo in a more anterior position to the endogenous exocrine pancreas (arrow, C, D). *trypsin*, a marker of exocrine function, is expressed in the exocrine pancreas from 48 hpf (arrowhead, E, F). *4C1* mutants express *trypsin* in an ectopic domain on the left side of the digestive tract anterior to the reduced endogenous exocrine pancreas (arrowhead, G, H).

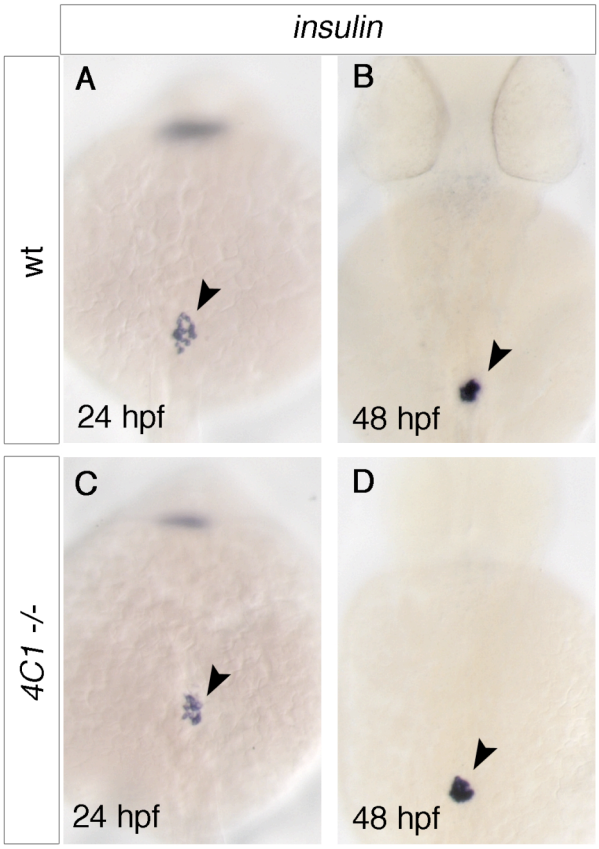


Figure 4.7 4C1 is dispensable for endocrine pancreas formation

In situ hybridisation analysis of *insulin* expression, dorsal views, anterior to top. Wild type embryos express *insulin* in the β-cells of the endocrine pancreas, which forms a single islet at 24 and 48 hpf (arrowhead, A, B). Analysis of *4C1* mutants reveals no defects in *insulin* expression at both stages (arrowhead, C, D).

forming endoderm from 24 hpf onwards (Fig. 4.7, A - D) confirming that 4C1 is not required for specification or morphogenesis of the endocrine pancreas in zebrafish.

4.5 4C1 is required for LPM differentiation and morphogenesis

The hepatic and pancreatic morphogenetic defects, as well as those observed in the ductal system of *4C1* mutants, could be the result of defects in the neighbouring LPM, which in zebrafish is the source of signals required for organ specification and outgrowth. In addition, asymmetric LPM migration directs digestive tract looping and initial analysis of *4C1* mutant embryos suggested that gut looping is defective in these embryos (Fig. 5.2). This raises the possibility that LPM formation is disrupted in *4C1* mutants resulting in the observed endodermal defects. To confirm that 4C1 is required for intestinal looping, I further analysed digestive tract morphogenesis at 24, 30 and 48 hpf by *foxA1* expression. At 24 hpf, endoderm morphology is indistinguishable between wild type embryos and *4C1* mutants (Fig. 4.8, A, D). However, by 30 hpf, when the digestive tract looping has been initiated in wild type embryos (Fig. 4.8, B), the digestive tract remains midline in *4C1* mutants (Fig. 4.8, E). Subsequently, gut looping in *4C1* mutants at 48 hpf occurs, however the displacement of the digestive tract appears less efficient in *4C1* mutants than in wild type embryos at the same stage (Fig. 4.8, compare, C, F).

Digestive tract looping results from asymmetric migration of the neighbouring LPM; between 24 and 30 hpf, the left lateral plate migrates dorsal to the foregut while the right lateral plate migrates ventrolaterally, resulting in looping of the foregut to the left side of the midline (Fig. 4.9, A, E). This migration is thought to be tightly linked to the establishment of epithelial polarity within the LPM (Horne-Badovinac et al., 2003). Failure of digestive tract looping in *4C1* mutants may be the result of defects

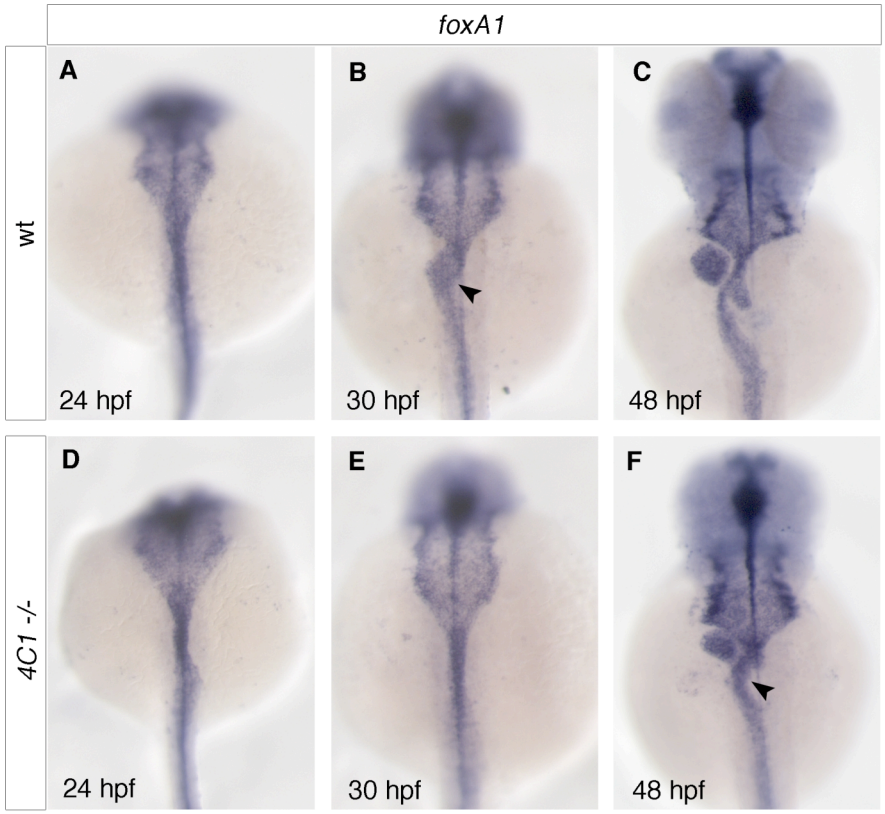


Figure 4.8 4C1 is required for digestive tract looping

In situ hybridisation analysis examining digestive tract morphogenesis by *foxA1* expression in wild type and *4C1* mutant embryos. (A-C) The endoderm in wild type embryos has formed a midline rod at 24 hpf (A); by 30 hpf leftward looping of the gut occurs at the level of the budding liver (arrowhead, B), which is more pronounced at 48 hpf (C). Endodermal rod formation appears unaffected in *4C1* mutants at 24 hpf (D), however, gut looping fails to occur at 30 hpf, and the digestive tract remains midline (E). By 48 hpf, gut looping has been initiated in *4C1* mutants (arrowhead, F). Dorsal views, anterior to top.

in epithelial polarisation and/or failed migration of the LPM. Transverse sections of the LPM in *4C1* mutants demonstrated that the polarised epithelial structure of the LPM is disrupted. The LPM in *4C1* mutants appears to be comprised mostly of cuboidal cells, and fails to adopt a squamous/columnar organisation (Fig. 4.9, B-D, F-H). Analysis of aPKCζ and aPKCλ expression, which is heightened at the apical membranes in the LPM at 30 hpf in wild type embryos (Fig. 4.9, E, E') suggests that in *4C1* mutant embryos, aPKC may not be heightened at the apical membrane of the LPM as observed in wild type embryos, but is expanded laterally (Fig. 4.9, F-H'); alternatively, this mild disruption to aPKC localisation may result from disorganisation of cells within the LPM. Further, LPM migration in *4C1* mutant embryos is defective. 47% of embryos displayed partial migration of the LPM lateral to the developing intestine, (n=51, Fig. 4.9, B, F), 43% displayed symmetric LPM migration dorsal to the gut (n=51, Fig. 4.9, C, G), while only a small number of embryos displayed partial asymmetric migration (10%, n=51, Fig. 4.9, D, H). In all cases, the digestive tract remains midline, although it is interesting to note that in a subset of embryos where LPM migration has failed the liver bud is located asymmetrically on the left side of the embryo (27%, n=51, Fig. 4.9, C, G). This suggests that LPM differentiation is disrupted in *4C1* mutants, resulting in LPM migration defects. These LPM defects may be responsible for the failure in gut looping in *4C1* mutants, in addition to the defects observed in hepatic and pancreatic organogenesis.

4.6 *4C1* is required for LPM gene expression and the formation of LPM-derived structures.

I observe defects in both endodermal and mesodermal tissues in *4C1* mutants – morphogenesis of liver and pancreas formation appears disrupted, and differentiation and morphogenesis of the neighbouring LPM is perturbed. To determine whether the hepatic and pancreatic defects observed in *4C1* mutants are the result of primary defects within the LPM, I examined the expression of liver and pancreas promoting factors in the LPM, in addition to the formation of additional LPM-derived structures. *prt/wnt2bb* is expressed in the LPM and is required for hepatoblast specification in the adjacent endoderm (Ober et al., 2006). Preliminary analysis at 24 hpf revealed a mild reduction of *prt/wnt2bb* expression in presumptive 4C1 mutants when compared to wild type embryos (Fig. 4.10, A, B) highlighting a possible cause for the reduced hepatic progenitor population in *4C1* mutants.

Further, as I observe defects in morphogenesis and outgrowth of the exocrine pancreas in *4C1* mutants I examined expression of *meis3* in the LPM, which is required for outgrowth of the exocrine pancreas in zebrafish (Manfroid et al., 2007). At 30 hpf, in wild type embryos, *meis3* is expressed medially in the LPM as a result of asymmetric migration of the lateral plates at this stage (Fig. 4.10, C). In *4C1* mutants, I was unable to detect medial *meis3* expression (Fig. 4.10, E). It is possible that *meis3* is expressed bilaterally in the LPM in *4C1* mutants, however, this expression may be obscured by bilateral *meis3* expression in the overlying neural tissue. Therefore, transverse sections through *4C1* mutant embryos at the level of the organ-forming region are required to determine whether *meis3* expression is present in the LPM. At 48 hpf, wild type embryos maintain *meis3* expression in the LPM surrounding the exocrine pancreas (Fig. 4.10, D). Similarly to analysis at 30 hpf, I was unable to detect this medial *meis3* expression in *4C1* mutants (Fig. 4.10, F). Preliminary analysis of transverse sections through the organ-forming region of *4C1*

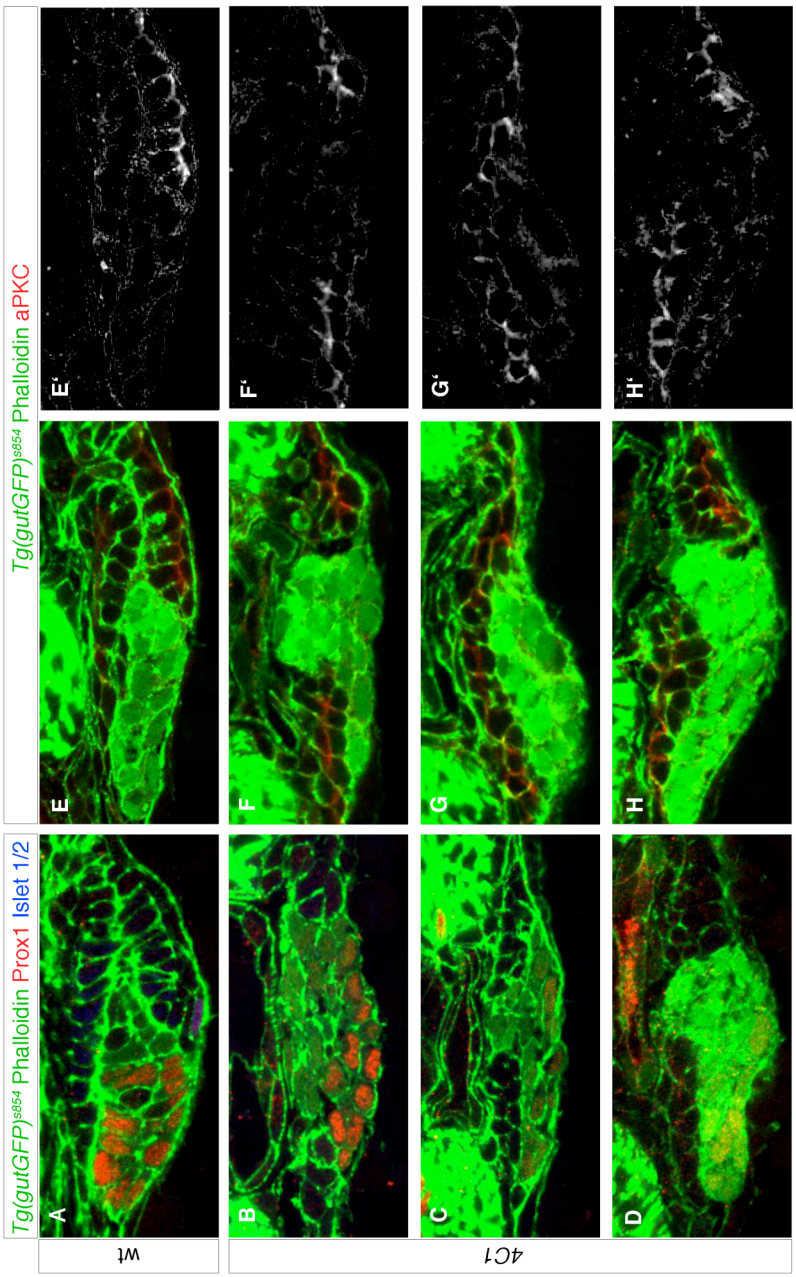

Tg(gutGFP)s854 Phalloidin Prox1 Islet 1/2
Tg(gutGFP)s854 Phalloidin aPKC
A
B
C
D
E
F
G
H
E'
F'
G'
H'
wt
4C1

Figure 4.9 4C1 is required for epithelial differentiation and concomitant migration of the LPM
(A-H) Transverse sections at the liver level of *Tg(gutGFP)*^{s854} wild type and *4C1* mutant embryos, stained with (A-D) Phalloidin (green), α-Prox1 (red) and α-Islet1/2 (blue), or (E-H) Phalloidin (green) and α-aPKC (red, with E'-H' showing aPKC staining only for clarity). In wild type embryos at 30 hpf, the LPM represents bilateral, polarised epithelia comprised of columnar and squamous cells that have migrated asymmetrically (A, E). In *4C1* mutants, the LPM is comprised of cuboidal cells, and fails to migrate correctly, with the majority of embryos exhibiting either partial lateral migration (B, F), or symmetric migration dorsally to the hepatic endoderm (C, G). A subset of embryos exhibit partial asymmetric migration (D, H). (E-H) In wild type embryos, aPKC is heightened at the apical membranes of the LPM (E, E'). In contrast, in *4C1* mutants, aPKC expression appears to extend to more lateral domains of the cells (F-H').

mutants (data not shown), in addition to morphological analysis of the digestive system by examining *foxA1* expression (Fig. 4.8), suggests that LPM migration is initiated by 48 hpf. Therefore, if *meis3* expression was present in *4C1* mutants it is likely that medial expression would be seen; the absence of this expression domain suggests that *4C1* is required for *meis3* expression in the LPM, highlighting a possible mechanism for failure of correct exocrine pancreas outgrowth in these mutants.

The previous data point to a role for *4C1* within the LPM, therefore I examined the formation of representative LPM-derived structures. *4C1* mutants display heart oedema, therefore I analysed early heart formation by expression of *cmlc2*, which is expressed in the forming heart. At 24 hpf in wild type embryos, *cmlc2* is expressed in the cardiomyoctes forming the heart cone (Fig. 4.11, A; Yelon et al., 1999), while in *4C1* mutants the number of cells expressing *cmlc2* is severely reduced (Fig. 4.11, B), suggesting that *4C1* is required for the correct specification and morphogenesis of this LPM-derived tissue. Further, due to defects observed in fin bud formation in *4C1* mutants, I analysed expression of *tbx5*, which is essential for fin bud formation and outgrowth (Fig. 4.11, C, D; Ahn et al., 2002). Interestingly, I observed reduced *tbx5* expression in the fin buds of *4C1* mutants at 30 and 48 hpf (Fig. 4.11, E, F) further suggesting a defect in initiation or maintenance of genes required for the formation of LPM-derived tissues.

To confirm the defects observed in fin bud development in *4C1* mutants, I analysed expression of *dhand*, which is expressed in a number of LPM derivatives, including the heart and presumptive fin buds (Yelon et al., 2000). Preliminary analysis reveals a downregulation of *dhand* in the presumptive fin buds of *4C1* mutants at 24, 30 and 48 hpf, however, levels of *dhand* expression appear unaffected in the LPM adjacent to the organ-forming endoderm at these stages (data not shown). Further analysis of

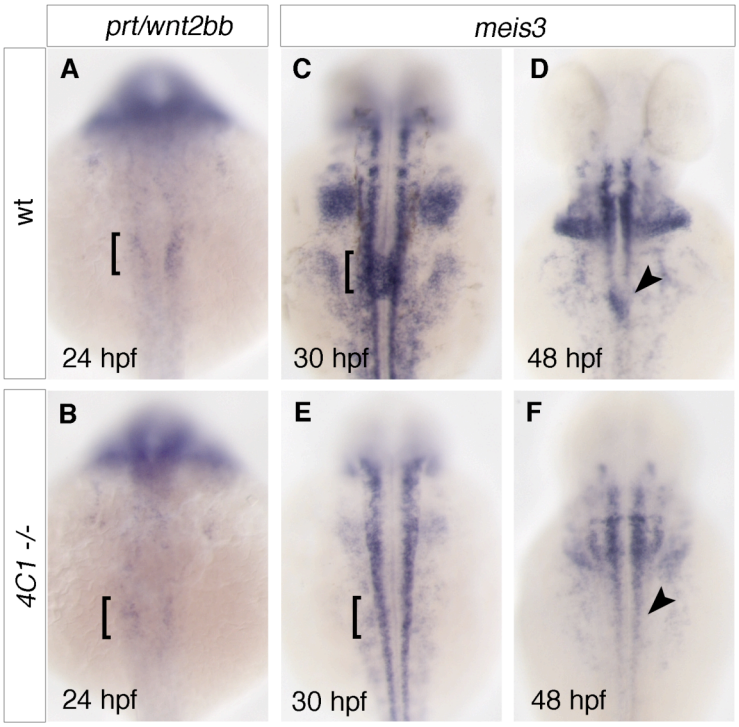


Figure 4.10 4C1 is required for LPM gene expression necessary for endodermal organogenesis.

In situ hybridisation analyses of *prt/wnt2b* and *meis3* expression, dorsal views, anterior to top. Wild type embryos express *prt/wnt2bb* in the LPM neighbouring the organ-forming endoderm at 24 hpf (bracket, A). However, *4C1* mutant embryos exhibit a mild reduction of *prt/wnt2bb* expression in this tissue (bracket, B). *meis3* is expressed medially in the LPM of wild type embryos at 30 hpf and 48 hpf (bracket, C, arrowhead, D). In *4C1* mutants *meis3* expression appears absent at both 30 and 48 hpf (bracket, E, arrowhead, F).

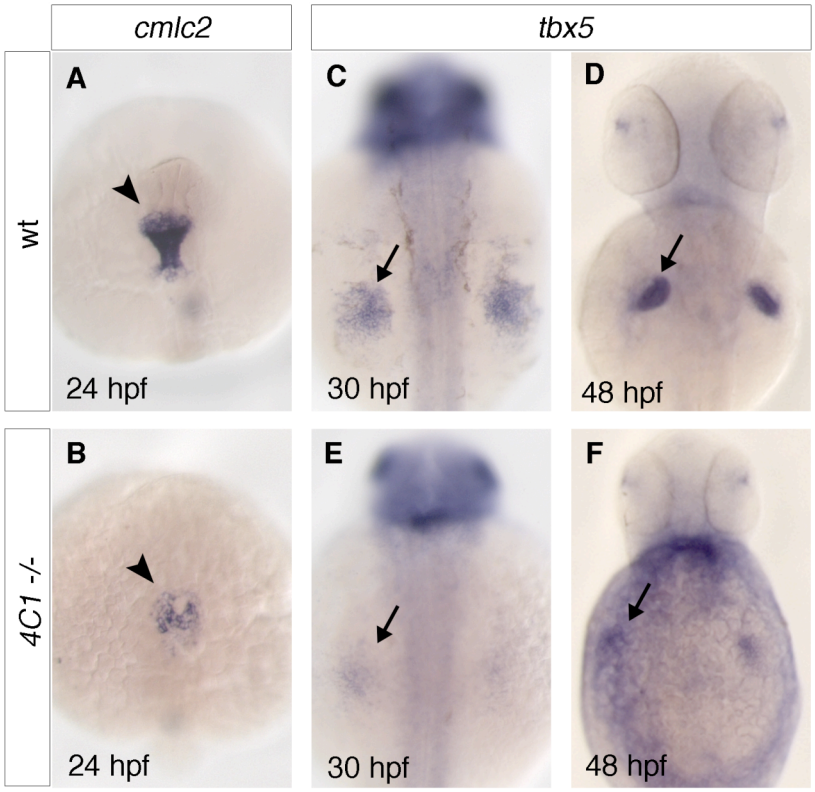


Figure 4.11 4C1 is required for gene expression in LPM-derived structures

In situ hybridisation analyses of *cmlc2* and *tbx5* expression in wild type and *4C1* mutant embryos; dorsal views, anterior to top. (A-B) Wild type embryos express *cmlc2* in the heart cone at 24 hpf (arrowhead, A). *4C1* mutants exhibit reduced *cmlc2* expression (arrowhead, B). (C-F) *tbx5* is expressed the fin buds at 30 and 48 hpf (arrow, C, D). *4C1* mutants have severely reduced levels of *tbx5* expression in the fin buds at these stages (arrows, E, F).

dhand expression will be required to determine the significance of the differential regulation of *dhand* in different LPM derivatives.

Together, these results suggest that 4C1 is required for the mesodermal expression of genes necessary for the development of LPM-derived organs, supporting a primary role for *4C1* in the mesoderm in hepatic and pancreatic organogenesis.

4.7 Positional cloning of 4C1

To isolate the lesion underlying the *4C1* phenotype, I initiated a positional cloning project. Polymorphic mapping strains were generated crossing *4C1 Tg(gutGFP)*^{s854} heterozygous carrieris with WIK wild type fish. Unfortunately, these lines were not sufficiently polymorphic, thus new mapcrosses with more suitable polymorphisms using the Ekkwill wild type line have been generated, and genetic linkage analysis to the *4C1* locus is currently in process.

4.8 Discussion

4C1 – roles in hepatic development – requirements in patterning and morphogenesis

Here I show a requirement for *4C1* in specification of the correct number of hepatoblasts and subsequent hepatic morphogenesis. Interestingly, *4C1* does not appear to play a role in timely specification or onset of differentiation of the liver. I have shown that loss of 4C1 function results in a reduction of the number of hepatoblasts specified at 24 hpf, and that the subsequent aggregation of these hepatoblasts to form a liver bud is disrupted, with Prox1-positive cells found in more posterior positions in *4C1* mutant embryos. This raises the intriguing possibility that *4C1* controls the migration of these cells to form the liver bud. Work in mouse has

demonstrated that Prox1 is required for migration of hepatoblasts (Sosa-Pineda et al., 2000). However, as Prox1 expression is present in *4C1* mutants, it is unlikely that this accounts for the migration defects observed in *4C1* mutants. Strikingly, at later stages it appears that the liver in *4C1* mutants is located in more anterior positions than in wild type embryos, suggesting that the subsequent migration and positioning of these cells is defective, possibly as a consequence of altered hepatic duct morphogenesis. Currently, the mechanisms by which bud formation progresses in zebrafish are unclear, therefore *4C1* mutants provide an interesting model to further our understanding of these processes.

The reduction in liver size in *4C1* mutants may not result from reduced proliferation, but may instead be due to a smaller pool of progenitors from which the liver will form. Therefore, it will be important to determine hepatoblast cell number at 22 hpf and 24 hpf, in addition to assessing the role of *4C1* in cell proliferation and survival in the liver to determine whether a single cause or combination of the above results in the hypoplastic liver observed in *4C1* mutants. At later stages of hepatic development, the liver remains severely reduced in size in *4C1* mutants and this difference in size becomes more pronounced over time, raising the possibility that *4C1* is regulating genes important for liver growth. It remains to be seen whether this is a requirement in the regulation of cell cycle gene expression cell-autonomously within the liver itself, or whether it is via the regulation of genes expressed in neighbouring tissue required for promoting organ growth.

4C1 does not appear to be required for the onset of hepatocyte differentiation, although to further support this hypothesis analysis of early hepatic differentiation

genes such as *cp* at the time of onset around 34 hpf is needed. It will also be important to examine the expression of later functional differentiation genes. In *hdac1* mutants, the liver is able (albeit with a delay) to progress to a state of differentiation whereby it expresses *cp*, but fails to express the later differentiation marker, *vitDbp*, suggesting that while intermediate stages of differentiation can be reached, functional differentiation fails to occur within the time points analysed. Therefore, assessing expression of genes such as *vitDbp* and *lfabp* will determine whether in *4C1* mutants hepatic differentiation at 48 hpf is able to translate into functional hepatocyte differentiation at later stages. Interestingly, formation of the intrahepatic biliary network, which is closely related to hepatocyte differentiation, appears perturbed in *4C1* mutants, suggesting that the differentiation of biliary epithelial cells is disrupted. Therefore it will be important to determine whether defects in functional differentiation are specific to formation of the intrahepatic biliary system by examining expression of factors such as *onecut1* and onecut3 (Matthews et al., 2008; Matthews et al., 2004), and whether these defects are independent of functional hepatocyte differentiation.

In addition, I observe dysmorphic extrahepatopancreatic duct formation in *4C1* mutants. Analysis of extrahepatic duct formation revealed that although 2F11 does become heightened in the duct at later time points, patterning of the duct appears perturbed. I observe an apparent increase in the amount of ductal tissue, with an ectopic duct connecting the ectopic pancreatic bud to the foregut, in addition to connecting the liver and endogenous exocrine pancreas to the digestive tract. Currently, little is known about the origin of extrahepatopancreatic ductal tissue, or the processes underlying the emergence of this duct from the digestive tract. It is

interesting to speculate whether organ anlage direct extrahepatopancreatic duct formation with respect to establishing a ductal connection, or whether formation of ductal tissue direct organ positioning. Due to the expansion in ductal tissue and increased number of organ anlage in *4C1* mutants, this line provides a good model to study the role of the liver and pancreas in directing duct formation, in addition to the identification of novel genes involved in this process.

Fgf10 signalling in the mesoderm has been shown to be required for proper maintenance of hepatic, pancreatic and ductal fates, with embryos defective in Fgf10 function exhibiting defects in morphogenesis of the extrahepatic duct, and an expansion of Prox1-positive cells in the ductal region in conjunction with ectopic expression of hepatic genes in the proximal pancreas, and vice versa (Dong et al., 2007). *4C1* mutants exhibit ectopic Prox-1 positive cells in the ductal region at 48 hpf, and it is interesting to speculate that 4C1 acts downstream of Fgf10 to mediate repression of Prox1 expression in the extrahepatopancreatic duct. Interestingly, depletion of *Hes1* in mouse results in the ectopic differentiation of exocrine pancreas from biliary epithelial cells (Sumazaki et al., 2004). Therefore, the ectopic exocrine cells observed in *4C1* mutants may result from defects in Notch signalling in the duct required to repress pancreatic differentiation in this tissue.

Requirements for 4C1 in pancreas morphogenesis

4C1 appears to be required for exocrine pancreas growth, with *4C1* mutants displaying a reduction in exocrine cell number. Analysis of the TALE-box protein *meis3* revealed that this gene is likely absent at 30 and 48 hpf, the time at which it has been implicated in growth of the exocrine pancreas (Manfroid et al., 2007). Therefore, it is possible that the reduction in size of the exocrine pancreas anlage is

due to an absence of *meis3* in the LPM. It will be important to determine whether injection of *meis3* mRNA into *4C1* mutant embryos is sufficient to rescue exocrine pancreas hypoplasia.

Work in zebrafish has shown that Prox1-positive hepatoblasts are specified at around 22 hpf, and subsequently aggregate anteriorly on the left side of the alimentary canal, to form a liver bud (Field et al., 2003b). The Prox1 and Ptf1a-positive cells of the exocrine pancreas are initiated at around 34 hpf, slightly posterior to the liver forming region, and these cells grow out to the right side of the endoderm, forming the exocrine pancreas (Field et al., 2003a; Lin et al., 2004). In *4C1* mutants, I observe a dysmorphic exocrine pancreatic domain that subsequently gives rise to two organ anlagen, one on the right side of the embryo in the correct position and one on the left side of the embryo, posterior to the liver. This phenotype could arise from a number of different causes. The ectopic exocrine population may result from defective exocrine migration and growth; alternatively they may represent additional cells that have been mis-specified from the foregut, or possibly form at the expense of the liver

Due to the observed defects in hepatic bud formation in *4C1* mutants, possibly through defective migration, this suggests that the formation of ectopic exocrine tissue in *4C1* mutants is also the result of migration defects. In wild type embryos, exocrine tissue is initiated on the left side of the embryo, and subsequently migrates and/or proliferates towards the right side. It is possible that in *4C1* mutants the initial specification of the exocrine pancreas is unaffected, however subsequent morphogenesis is impaired resulting in mispositioning of a subset of exocrine cells on the opposite side of the digestive tract. Interestingly, *4C1* mutants exhibit an apparent posterior expansion of Prox1-positive hepatoblasts. It is interesting to speculate that in *4C1* mutants the ectopic Prox1-positive cells in posterior positions become mis-

specified as exocrine pancreas or extrahepaotpancreatic duct, resulting in morphogenetic defects in formation of these structures. This would suggest an overall increase in exocrine pancreas tissue and extrahepatopancreatic duct tissue, and while the duct domain appears expanded in *4C1* mutants, an increase in exocrine cell number seems unlikely, supporting the theory *4C1* is involved in the morphogenetic processes required for bud formation and growth of the exocrine pancreas.

To understand the morphological events underlying the exocrine pancreas phenotype in *4C1* mutants it will be necessary to further examine the formation of the exocrine pancreas at earlier stages. It will be important to determine whether exocrine pancreas initiation occurs as wild type in *4C1* mutants, and that subsequent morphogenesis, proliferation and migration of these cells fail to occur, or whether the initial positioning of the newly specified exocrine cells is defective. In order to determine this, either use of in situ hybridisation or a transgenic line expressing GFP under the control of the *ptf1a* promoter (Godinho et al., 2005) would allow real time imaging of the specification and morphogenesis of the exocrine pancreas in *4C1* mutants.

4C1 – Roles in the endoderm versus the mesoderm

The data presented here have revealed a requirement for *4C1* in hepatic as well as pancreatic organogenesis in zebrafish. The formation of both organs requires interactions between the endoderm and the overlying mesoderm, which raises the question of whether *4C1* is required in the endoderm or mesoderm in this process. Several lines of evidence point to a requirement for *4C1* primarily in mesodermal tissue.

Firstly, the mesoderm is important for providing signals essential for the specification of both the liver and the pancreas. Interestingly, defects are observed in specification of both the liver and pancreas – hepatoblast specification occurs in a reduced number of cells, and this may be the result of a mild reduction in expression of instructive factors such as *prt/wnt2bb* in the neighbouring LPM. In addition, the TALE-box protein *meis3* is required for exocrine pancreas growth (Manfroid et al., 2007) and its expression appears absent in *4C1* mutants at 30 and 48 hpf. Altogether, it is possible that the defects in hepatic specification and pancreatic growth are due to a reduction in expression of mesodermally-derived promoting factors.

Studies in zebrafish have demonstrated that *meis3* expression in the LPM is regulated by Fgf signalling (Manfroid et al., 2007), and Fgf signalling is also important in formation of the extrahepatopancreatic duct (Dong et al., 2007). Due to the absence of *meis3* gene expression in the LPM of *4C1* mutants, and the defects observed in extrahepatopancreatic duct formation, it is interesting to speculate that *4C1* encodes a downstream effector of Fgf signalling.

Secondly, the LPM displays defects in epithelial morphology in addition to altered expression of epithelial polarity markers, resulting in disrupted LPM migration in *4C1* mutants. It is possible at this stage that the LPM is not correctly positioned with regard to the presumptive organ-forming endoderm, and the subsequent defects in specification and growth result from the misplaced source of these factors, resulting in less efficient signalling. Interestingly, asymmetric LPM migration and directed gut looping is required to regulate organ chirality (Horne-Badovinac et al., 2001; Horne-Badovinac et al., 2003), suggesting that the bilateral positioning of the exocrine pancreas in *4C1* mutants may be a consequence of the observed defects in gut looping. However, mutants that display defects in LPM migration also exhibit

randomisation of organ chirality for both liver and pancreas. *4C1* mutants do not exhibit defects in liver chirality, therefore it is unlikely that the mild gut looping defects alone can account for the bilateral exocrine pancreas.

Thirdly, in addition to defects in hepatic and pancreatic organogenesis, LPM derivatives such as the heart and fin buds appear to be affected in *4C1* mutants.

Although the data presented here suggest a primary requirement for *4C1* in the mesoderm, studies have shown that the endoderm itself is required to pattern the adjacent mesoderm (Manfroid et al., 2007) raising the intriguing possibility that *4C1* is acting in the endoderm to direct patterning of the mesoderm allowing hepatic and pancreatic organogenesis to occur. Mosaic experiments, transplanting wild type cells into the endoderm or mesoderm of *4C1* mutants, and vice versa will address these questions. By replacing the endoderm of wild type embryos with *4C1* mutant endoderm and subsequently examining endodermal organogenesis, it will be possible to elucidate whether loss of 4C1 function in the mesoderm is the primary defect giving rise to the phenotypes observed in *4C1* mutants.

Here, I have presented requirements for *4C1* in hepatic precursor formation and hepatic and pancreatic morphogenesis. Preliminary analyses suggest a primary requirement for *4C1* in the mesoderm to drive organogenesis. Identification of the lesion underlying this phenotype will allow subsequent analysis of the mechanisms by which *4C1* directs liver and pancreas formation, uncovering new mechanisms directing digestive system organogenesis in zebrafish.

Chapter 5

Conclusions

Endodermal organogenesis is a process that requires a combination of tightly controlled gene expression necessary for specification and differentiation of different cell types, coupled with morphological changes and cell movements that allow the generation of different tissues from common progenitors. The aim of this project was to identify novel factors required in the processes underlying liver organogenesis in the context of the developing zebrafish digestive system by the phenotypic characterisation and positional cloning of two mutant lines, and to place these two genes within the network of existing mechanisms required for digestive tract organogenesis. The requirements I have identified for these two genes in hepatic development, based on the cumulative data presented in this thesis, are outlined in Figure 5.1.

Here I contrast the endodermal phenotypes observed in these two mutant lines, and compare the relative similarities and differences in their modes of action in the processes underlying liver as well as pancreas organogenesis.

5.1 Similarities and differences between the roles of *hdac1* and *4C1* in hepatogenesis.

hdac1 and *4C1* mutants exhibit similar phenotypic defects at 48 hpf characterised by a hypoplastic liver. Further investigation has highlighted significant differences in the requirements for both these genes in development of the liver during embryogenesis. Analysis of *hdac1* mutants revealed a primary requirement for *hdac1* in timely specification of hepatoblasts in addition to specification of the correct number of hepatoblasts, whereas *4C1* appears to be required for correct number of hepatoblast progenitors specified at 24 hpf and positioning of these cells. Both genes appear to directly or indirectly regulate expression of the hepatic specification factor *prt/wnt2bb*

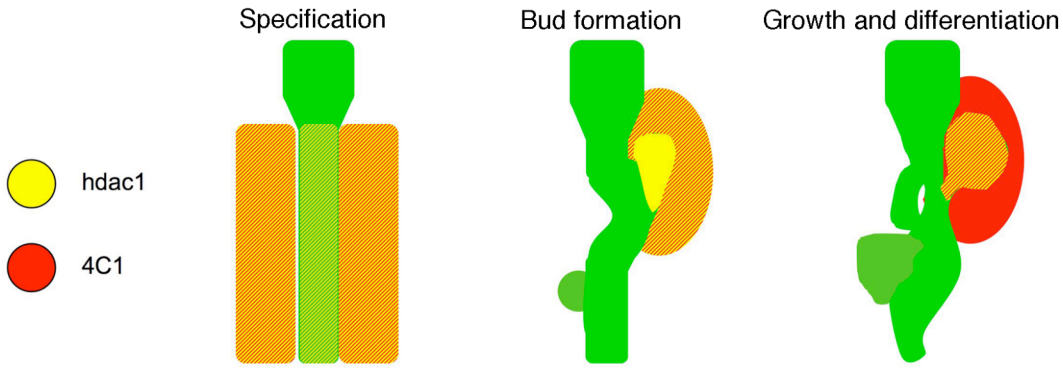


Fig. 5.1 The proposed requirements for *hdac1* and *4C1* in zebrafish hepatic organogenesis
Liver formation. Data presented within this report suggests that Hdac1 is required within the endoderm and mesoderm for hepatic specification and subsequent bud formation. Additionally, Hdac1 is required cell-autonomously within the endoderm for hepatic differentiation and subsequent growth. 4C1 is proposed to act within the mesoderm for hepatic specification and bud formation, however may be required in either the endoderm or mesoderm for hepatic growth.

in the adjacent mesoderm. My work indicates that in addition to regulating factors in the neighbouring mesoderm, *hdac1* may play a cell-autonomous role within the endoderm in promoting hepatic competence accounting for the more severe phenotype observed in hepatic specification than that observed in *4C1* mutants. Conversely, my data suggests that *4C1* is acting primarily within the mesoderm. It is possible that the mild reduction in hepatic progenitors specified in *4C1* mutants is due solely to reduction in mesodermal *prt/wnt2bb* expression in the mesoderm, however, it will be important to determine whether the expression of other factors required for hepatic induction are also altered.

Additionally, in *hdac1* mutants, foregut tissue forms at the expense of the liver and pancreas demonstrating that Hdac1 promotes hepatoblast formation from of the adjacent non-hepatic foregut endoderm, suggesting Hdac1 is required for the genetic induction of organ specification. Quantification of cell number within the foregut endoderm has not been determined in *4C1* mutants, however, the foregut does not appear increased in size, suggesting that *4C1* is not involved in segregating hepatic and pancreatic fate from foregut endoderm.

I have demonstrated that *hdac1* is required for timely hepatic differentiation, whereas initial stages of this process appear to proceed independent of *4C1* function, although it will be important to determine whether 4C1 is required for functional differentiation of hepatocytes at later stages of development. However, both genes appear to be required for the differentiation and elaboration of the intrahepatic biliary system. Furthermore, I have demonstrated a cell-autonomous role for Hdac1 in hepatic development within the endoderm, raising the possibility that Hdac1 is primarily

acting within the endoderm to confer hepatic competence on the foregut allowing it to respond to specification as well as differentiation signals.

Both mutants exhibit a reduction in liver size at 48 hpf. Hdac1 has been shown to regulate cell proliferation in other tissues within the developing zebrafish embryo, and here I demonstrated that Hdac1 is also required to maintain levels of proliferation within the liver and adjacent foregut at early stages. It is possible that proliferation defects account for the reduction in size of the liver bud in *4C1* mutants, although the reduction in liver size could result solely from the initial deficit in the progenitor population, or a combination of these factors.

5.2 Distinct requirements for Hdac1 and 4C1 in pancreatic development

Both mutant lines exhibit distinct defects in formation of the exocrine pancreas. Exocrine pancreas specification in *hdac1* mutants occurs with a delay of about 40 hours, suggesting that *hdac1* is required for timely specification of this tissue, in addition to a role in the subsequent growth and differentiation. Conversely, in *4C1* mutants it appears that exocrine pancreas specification may occur spatially and temporally correctly, but the morphogenesis of this organ is disrupted and an ectopic exocrine pancreas forms posterior to the liver. In summary, my data indicate that Hdac1 is predominantly required to initiate and/or maintain the molecular pathways underlying pancreas specification and differentiation at the correct time, whereas 4C1 is likely involved in regulating the morphogenesis and growth of this organ. These findings are in line with the observed requirements for Hdac1 and 4C1 in hepatic development suggesting conserved requirements between organs for Hdac1 in promoting temporal organ specification and differentiation, and for 4C1 in promoting organ growth and morphogenesis. An interesting exception to the role of Hdac1 in

organ specification and differentiation arises in its requirement in endocrine pancreas morphogenesis, as preliminary data suggest that *hdac1* may be required for aggregation of these cells to form the endocrine islet.

5.3 Outlook: Requirements for Hdac1 and 4C1 in the endoderm and mesoderm

Studies in mouse, chick and zebrafish have demonstrated the importance of interactions between the mesoderm and endoderm in promoting organogenesis. Interestingly, both mutant lines exhibit defects in expression of LPM derived genes necessary for organ specification and outgrowth, in addition to defects in LPM morphology, raising the question of in which tissue the primary requirements for Hdac1 and 4C1 are in endodermal organogenesis.

My data suggest it is likely that the organogenesis defects are caused by different mechanisms in these two mutant lines. I have demonstrated a cell-autonomous requirement in the endoderm for Hdac1 in liver development, and despite the reduction in mesodermally derived specification factors in *hdac1* mutants, it is likely that the primary requirement for Hdac1 is to provide hepatic competence within the endoderm. Previous studies have demonstrated that correct positioning of promoting factors in the LPM is important for directing organ growth (Huang et al., 2008). In *hdac1* mutants, due to LPM migration defects the positioning of *meis3*-expressing cells, required for exocrine pancreas outgrowth, in relation to the organ-forming endoderm appears to be defective. As *meis3* is required only for outgrowth and not specification of the exocrine pancreas, this suggests that Hdac1 regulates other genes in the mesoderm necessary for exocrine pancreas specification, or is required in the endoderm to allow cells to respond to specification signals, similar to its proposed requirement in hepatic specification.

Conversely, in *4C1* mutants, the primary defect in endodermal organogenesis is morphogenesis and organ growth. A number of factors expressed in the mesoderm required for both endodermal and mesodermal organ morphogenesis and growth are reduced. This, coupled with fact that timely hepatic and pancreatic specification and differentiation doesn't appear to be affected in *4C1* mutants suggests that the primary requirement for *4C1* is in the mesoderm to direct organ morphogenesis and growth. Altogether, my data point strongly to a requirement for *4C1* in the mesoderm, and a primary requirement for *hdac1* within the endoderm, for promotion of endodermal organogenesis.

In summary, I have demonstrated specific requirements for *hdac1* and *4C1* in endodermal organogenesis. While zebrafish with mutations in these genes display superficially similar phenotypic defects in liver development, in depth analysis revealed very distinct roles for each individual gene in formation of the liver within the context of the digestive system. Isolation of the lesion underlying the *4C1* phenotype promises to add to our understanding of the role of the mesoderm in liver development. Identification and detailed analysis of the downstream targets of both *hdac1* and *4C1* will further our understanding of the highly complex processes regulating hepatic organogenesis. Moreover, this study highlights the relevance of forward genetic screens in identifying novel genes, or novel roles for known genes, unravelling the gene regulatory networks underlying liver formation during embryogenesis.

Appendix 1

Isolation of zebrafish *vitamin D binding protein*

Aim

In mouse, *Albumin* is widely regarded as the earliest definitive hepatic marker, expressed in migrating hepatoblasts from E9.5 onwards (Cascio and Zaret, 1991). However, in zebrafish there is currently a lack of similar markers – genes expressed early in the liver such as *hhex* and *prox1* are not restricted to hepatic expression (Field et al., 2003b; Ober et al., 2006), and the earliest markers of hepatic differentiation, such as *cp* and *sePb*, are plasma proteins which are in addition expressed in the YSL (Korzh et al., 2001; Kryukov and Gladyshev, 2000). In zebrafish, an *albumin* gene has not been reported. Therefore, I aimed to identify the zebrafish homologue of murine *Albumin*, determining whether this gene is evolutionarily conserved, and providing us with an early definitive marker for the hepatic lineage in zebrafish.

Materials and methods

To identify regions of conservation between mouse and zebrafish, the coding sequence (CDS) of mouse *Albumin* (NM_009654) was used in a Blast search against the zebrafish genome. The zebrafish sequence with the most significant alignment was identified as NM_001002568, a 1610 bp mRNA, encoding an uncharacterised protein hypothesised to have vitamin D binding properties. This gene is situated on LG5, between 47.5 and 47.6 Mb. Subsequently, 2 sets of primers were designed based on regions of homology between the mouse *Albumin* and putative zebrafish *albumin*.

Primer	Sequence	Position
F1	GCAGAAAAGACTCTCCATTCC	340
F2	GAGGAAACTTTGTCTGGCTTC	407
R1	TAAGCAGCACTGTCATCATCA	1447
R2	GAGTCTCTCCTCCCTCCTTCT	1332

A 1108 bp fragment was amplified from 72 hpf *Tg(gutGFP)*^{s854} cDNA using primers F1 and R1. This fragment was cloned into the PCRII vector, using the Dual Promotor TA Cloning Kit (Invitrogen) Blunt end ligations were performed for 4 hours at 14°C in a volume of 10μl using 1μl of ligase, and the ligated plasmids were transformed into DH5α cells. To determine the direction of the insert, diagnostic digests were performed, and the cloned fragment was sequenced (Figure A1). In situ hybridisation probes were synthesised as previously described, BamHI was used to linearise the plasmid, and the antisense mRNA probe was transcribed using T7.

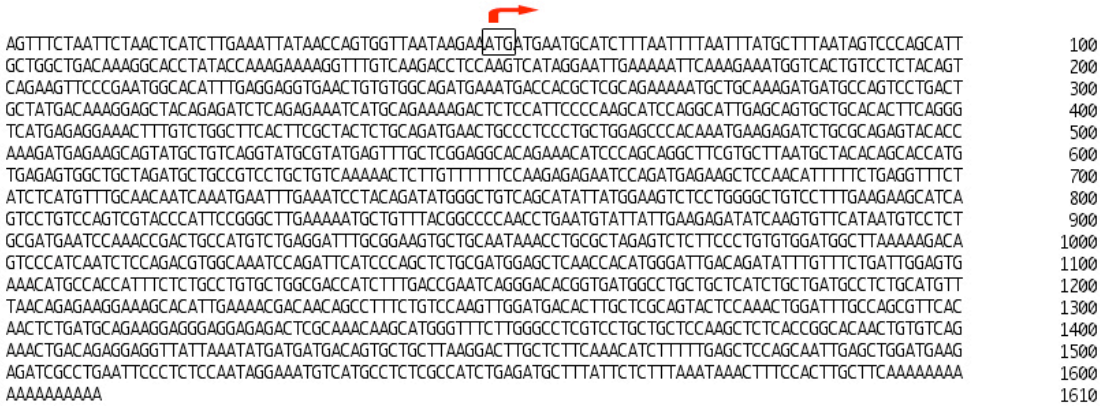


Figure A1 *vitamin D binding protein* coding sequence

Box and red arrow indicate open reading frame

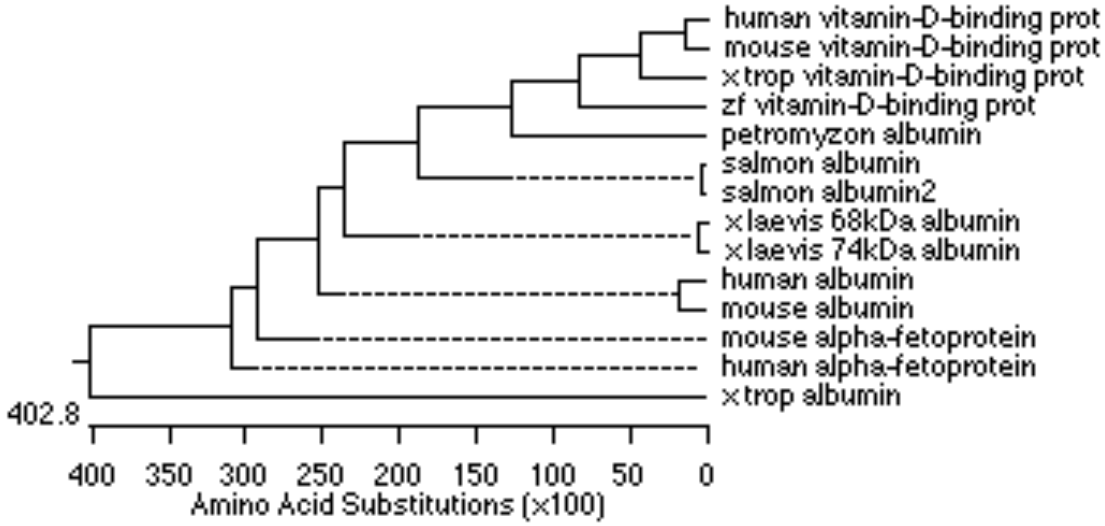


Figure A2 Phylogenetic analysis of zebrafish *vitamin D binding protein*

Sequence comparison between zebrafish *vitDbp* and related vertebrate proteins reveals that zebrafish *vitDbp,* the homologue of human, mouse and frog *Vitamin D binding protein,* is closely related to mammalian *Albumin*. Phylogenetic analysis demonstrates in mammals this gene derives from a common ancestor of both *Albumin* and α*-fetoprotein*.

Results and discussion

Using sequence homology, I have identified the closest zebrafish homologue of murine *Albumin*, the earliest definitive liver marker in mouse. However, phylogenetic comparison of the zebrafish gene isolated revealed the highest homology to mouse *Vitamin D binding protein*, which derives from a common ancestor of *Albumin* and *α-fetoprotein* in mouse (Figure A2; Haefliger et al., 1989). Together with predicted vitamin D-binding function, this suggests that in zebrafish the closest homologue to murine *Albumin* is *Vitamin D binding protein*. *Albumin, α-fetoprotein* and *Vitamin D binding protein* are part of the same gene family, and it has been demonstrated that these three genes are the result of 2 genome duplication events. The first genome duplication event occurred before the segregation of the mammalian/reptilian and amphibian divergence, resulting in primitive *Albumin* and *VDBP*. Subsequently, the duplication of the *Albumin* ancestor resulted in evolution of *Albumin* and *α-fetoprotein* – this divergence is predicted to have occurred after segregation of reptilian and amphibian lineages from mammals, hence the former do not contain *α-fetoprotein* within their genomes. However, some fish, such as salmon, contain an *Albumin* gene (Byrnes and Gannon, 1990), suggesting that zebrafish may have secondarily lost the *albumin* gene after the divergence of these two species.

Analysis of *vitamin D binding protein* expression in zebrafish reveals onset of expression at around 60 hpf (Fig. 3.7). This suggests that while *vitamin D binding protein* expression analysis is not useful for identifying early hepatic lineage in zebrafish, identification of this gene is highly useful for determining functional hepatic differentiation in older embryos.

Appendix 2

Comparison of hepatic and pancreatic gene expression in *hdac1*^{hi1618} and *hdac1*^{s436} alleles

		hdac1^{hi1618}	*hdac1*^{s436}
hhex	24 hpf	0% (n=17)	0% (n=17)
hepatoblast domain	30 hpf	87% (n=15)	88% (n=33)
cp	48 hpf	0% (n=23)	0% (n=15)
liver	72 hpf	6% (n=17)	19% (n=16)
	96 hpf	17% (n=23)	50% (n=18)
pitf1a	48 hpf	78% (n=32)	10% (n=20)
exocrine pancreas	72 hpf	100% (n=12)	100% (n=10)
insulin	24 hpf	0% (n=14)	39% (n=33)
single endocrine	30 hpf	19% (n=21)	31% (n=39)
pancreas	48 hpf	43% (n=14)	28% (n=44)

Comparison of phenotypic severity between the two *hdac1* alleles, *hi1618* and *s436,* examining hepatic (*hhex, cp*) and pancreatic (*pitf1a, insulin*) gene expression. Numbers presented indicate percentage of mutant embryos with gene expression in the liver (*hhex, cp*), expression in the exocrine pancreas (*pitf1a)*, and percentage of embryos with single endocrine pancreas cell clusters (*insulin*).

References

Adams, D. H. and Eksteen, B. (2006). Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. *Nat Rev Immunol* **6**, 244-51.

Ader, T., Norel, R., Levoci, L. and Rogler, L. E. (2006). Transcriptional profiling implicates TGFbeta/BMP and Notch signaling pathways in ductular differentiation of fetal murine hepatoblasts. *Mech Dev* **123**, 177-94.

Ahn, D. G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. *Nature* **417**, 754-8.

Alexander, J. and Stainier, D. Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol* **9**, 1147-57.

Allende, M. L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N. and Hopkins, N. (1996). Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. *Genes Dev* **10**, 3141-55.

Amsterdam, A., Nissen, R. M., Sun, Z., Swindell, E. C., Farrington, S. and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci USA* **101**, 12792-7.

Angelo, S., Lohr, J., Lee, K. H., Ticho, B. S., Breitbart, R. E., Hill, S., Yost, H. J. and Srivastava, D. (2000). Conservation of sequence and expression of Xenopus and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development. *Mech Dev* **95**, 231-7.

Aoki, T. O., David, N. B., Minchiotti, G., Saint-Etienne, L., Dickmeis, T., Persico, G. M., Strähle, U., Mourrain, P. and Rosa, F. M. (2002). Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. *Development* **129**, 275-86.

Archer, S. Y., Johnson, J. J., Kim, H. J. and Hodin, R. A. (2001). p21 gene regulation during enterocyte differentiation. *J Surg Res* **98**, 4-8.

Argenton, F., Zecchin, E. and Bortolussi, M. (1999). Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. *Mech Dev* **87**, 217-21.

Bagnat, M., Cheung, I. D., Mostov, K. E. and Stainier, D. Y. (2007). Genetic control of single lumen formation in the zebrafish gut. *Nat Cell Biol* **9**, 954-60.

Beaudry, J. B., Pierreux, C. E., Hayhurst, G. P., Plumb-Rudewiez, N., Weiss, M. C., Rousseau, G. G. and Lemaigre, F. P. (2006). Threshold levels of hepatocyte nuclear factor 6 (HNF-6) acting in synergy with HNF-4 and PGC-1alpha are required for time-specific gene expression during liver development. *Mol Cell Biol* **26**, 6037-46.

Begemann, G. and Ingham, P. W. (2000). Developmental regulation of Tbx5 in zebrafish embryogenesis. *Mech Dev* **90**, 299-304.

Biemar, F., Argenton, F., Schmidtke, R., Epperlein, S., Peers, B. and Driever, W. (2001). Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. *Dev Biol* **230**, 189-203.

Binamé, F., Lassus, P. and Hibner, U. (2008). Transforming growth factor beta controls the directional migration of hepatocyte cohorts by modulating their adhesion to fibronectin. *Mol Biol Cell* **19**, 945-56.

Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H. and McKnight, S. L. (1989). Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev* **3**, 1146-56.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-71.

Bogue, C. W., Ganea, G. R., Sturm, E., Ianucci, R. and Jacobs, H. C. (2000). Hex expression suggests a role in the development and function of organs derived from foregut endoderm. *Dev Dyn* **219**, 84-9.

Bort, R., Martinez-Barbera, J. P., Beddington, R. S. and Zaret, K. S. (2004). Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. *Development* **131**, 797-806.

Bort, R., Signore, M., Tremblay, K., Martinez Barbera, J. P. and Zaret, K. S. (2006). Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev Biol* **290**, 44-56.

Bossard, P. and Zaret, K. S. (1998). GATA transcription factors as potentiators of gut endoderm differentiation. *Development* **125**, 4909-17.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Burke, Z. and Oliver, G. (2002). Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. *Mech Dev* **118**, 147-55.

Byrnes, L. and Gannon, F. (1990). Atlantic salmon (Salmo salar) serum albumin: cDNA sequence, evolution, and tissue expression. *DNA Cell Biol* **9**, 647-55.

Calmont, A., Wandzioch, E., Tremblay, K. D., Minowada, G., Kaestner, K. H., Martin, G. R. and Zaret, K. S. (2006). An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. *Dev Cell* **11**, 339-48.

Calvo, D., Victor, M., Gay, F., Sui, G., Luke, M. P., Dufourcq, P., Wen, G., Maduro, M., Rothman, J. and Shi, Y. (2001). A POP-1 repressor complex restricts inappropriate cell type-specific gene transcription during Caenorhabditis elegans embryogenesis. *EMBO J* **20**, 7197-208.

Cano, D. A., Hebrok, M. and Zenker, M. (2007). Pancreatic development and disease. *Gastroenterology* **132**, 745-62.

Cascio, S. and Zaret, K. S. (1991). Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. *Development* **113**, 217-25.

Chen, J., Ruan, H., Ng, S. M., Gao, C., Soo, H. M., Wu, W., Zhang, Z., Wen, Z., Lane, D. P. and Peng, J. (2005). Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish. *Genes Dev* **19**, 2900-11.

Cheng, W., Guo, L., Zhang, Z., Soo, H. M., Wen, C., Wu, W. and Peng, J. (2006). HNF factors form a network to regulate liver-enriched genes in zebrafish. *Dev Biol* **294**, 482-96.

Chiba, T., Yokosuka, O., Arai, M., Tada, M., Fukai, K., Imazeki, F., Kato, M., Seki, N. and Saisho, H. (2004). Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. *J Hepatol* **41**, 436-45.

Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., de Wit, E., Fischer, B. E., van Steensel, B., Micklem, G. and Brand, A. H. (2006). Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. *Dev Cell* **11**, 775-89.

Chung, W. S., Shin, C. H. and Stainier, D. Y. (2008). Bmp2 signaling regulates the hepatic versus pancreatic fate decision. *Dev Cell* **15**, 738-48.
Chung, W. S. and Stainier, D. Y. (2008). Intra-endodermal interactions are required for pancreatic beta cell induction. *Dev Cell* **14**, 582-93.
Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M. and Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* **9**, 279-89.
Cirillo, L. A. and Zaret, K. S. (1999). An early developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA. *Mol Cell* **4**, 961-9.
Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C. E., Van der Smissen, P., Dietz, H. C., Courtoy, P. J., Rousseau, G. G. and Lemaigre, F. P. (2005). Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* **19**, 1849-54.
Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G. G. and Lemaigre, F. P. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**, 1819-28.
Clotman, F., Libbrecht, L., Gresh, L., Yaniv, M., Roskams, T., Rousseau, G. G. and Lemaigre, F. P. (2003). Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development. *J Hepatol* **39**, 686-92.
Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schütz, G., Babinet, C., Pontoglio, M., Yaniv, M. and Barra, J. (2002). Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129**, 1829-38.
Costa, R. H., Grayson, D. R. and Darnell, J. E. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. *Mol Cell Biol* **9**, 1415-25.
Cunliffe, V. T. (2004). Histone deacetylase 1 is required to repress Notch target gene expression during zebrafish neurogenesis and to maintain the production of motoneurones in response to hedgehog signalling. *Development* **131**, 2983-95.
Cunliffe, V. T. and Casaccia-Bonnefil, P. (2006). Histone deacetylase 1 is essential for oligodendrocyte specification in the zebrafish CNS. *Mech Dev* **123**, 24-30.
de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S. and van Kuilenburg, A. B. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* **370**, 737-49.
Decaens, T., Godard, C., de Reyniès, A., Rickman, D. S., Tronche, F., Couty, J. P., Perret, C. and Colnot, S. (2008). Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. *Hepatology* **47**, 247-58.
Defrances, M. C., Wolf, H. K., Michalopoulos, G. K. and Zarnegar, R. (1992). The presence of hepatocyte growth factor in the developing rat. *Development* **116**, 387-95.
Denson, L. A., McClure, M. H., Bogue, C. W., Karpen, S. J. and Jacobs, H. C. (2000). HNF3beta and GATA-4 transactivate the liver-enriched homeobox gene, Hex. *Gene* **246**, 311-20.
Dessimoz, J., Opoka, R., Kordich, J. J., Grapin-Botton, A. and Wells, J. M. (2006). FGF signaling is necessary for establishing gut tube domains along the anterior-posterior axis in vivo. *Mech Dev* **123**, 42-55.

Deutsch, G., Jung, J., Zheng, M., Lóra, J. and Zaret, K. S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* **128**, 871-81.

Dickmeis, T., Mourrain, P., Saint-Etienne, L., Fischer, N., Aanstad, P., Clark, M., Strähle, U. and Rosa, F. (2001). A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. *Genes Dev* **15**, 1487-92.

diIorio, P., Alexa, K., Choe, S. K., Etheridge, L. and Sagerström, C. G. (2007). TALE-family homeodomain proteins regulate endodermal sonic hedgehog expression and pattern the anterior endoderm. *Dev Biol* **304**, 221-31.

diIorio, P. J., Moss, J. B., Sbrogna, J. L., Karlstrom, R. O. and Moss, L. G. (2002). Sonic hedgehog is required early in pancreatic islet development. *Dev Biol* **244**, 75-84.

Dong, P. D., Munson, C. A., Norton, W., Crosnier, C., Pan, X., Gong, Z., Neumann, C. J. and Stainier, D. Y. (2007). Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. *Nat Genet* **39**, 397-402.

Dong, P. D., Provost, E., Leach, S. D. and Stainier, D. Y. (2008). Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. *Genes Dev* **22**, 1445-50.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z. et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.

Dufourcq, P., Victor, M., Gay, F., Calvo, D., Hodgkin, J. and Shi, Y. (2002). Functional requirement for histone deacetylase 1 in Caenorhabditis elegans gonadogenesis. *Mol Cell Biol* **22**, 3024-34.

Duncan, S. A. (2005). Generation of embryos directly from embryonic stem cells by tetraploid embryo complementation reveals a role for GATA factors in organogenesis. *Biochem Soc Trans* **33**, 1534-6.

Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat Genet* **34**, 53-8.

Farooq, M., Sulochana, K. N., Pan, X., To, J., Sheng, D., Gong, Z. and Ge, R. (2008). Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. *Dev Biol* **317**, 336-53.

Field, H. A., Dong, P. D., Beis, D. and Stainier, D. Y. (2003a). Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. *Dev Biol* **261**, 197-208.

Field, H. A., Ober, E. A., Roeser, T. and Stainier, D. Y. (2003b). Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol* **253**, 279-90.

Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. and Pavletich, N. P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401**, 188-93.

Foley, A. C., Gupta, R. W., Guzzo, R. M., Korol, O. and Mercola, M. (2006). Embryonic heart induction. *Ann N Y Acad Sci* **1080**, 85-96.

Fukuda-Taira, S. (1981). Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. *Journal of embryology and experimental morphology* **63**, 111-25.

Geisler, R., Rauch, G. J., Geiger-Rudolph, S., Albrecht, A., van Bebber, F., Berger, A., Busch-Nentwich, E., Dahm, R., Dekens, M. P., Dooley, C. et al.

(2007). Large-scale mapping of mutations affecting zebrafish development. *BMC Genomics* **8**, 11.
Glasgow, E. and Tomarev, S. I. (1998). Restricted expression of the homeobox gene prox 1 in developing zebrafish. *Mech Dev* **76**, 175-8.
Glozak, M. A. and Seto, E. (2007). Histone deacetylases and cancer. *Oncogene* **26**, 5420-32.
Godinho, L., Mumm, J. S., Williams, P. R., Schroeter, E. H., Koerber, A., Park, S. W., Leach, S. D. and Wong, R. O. (2005). Targeting of amacrine cell neurites to appropriate synaptic laminae in the developing zebrafish retina. *Development* **132**, 5069-79.
Goessling, W., North, T. E., Lord, A. M., Ceol, C., Lee, S., Weidinger, G., Bourque, C., Strijbosch, R., Haramis, A. P., Puder, M. et al. (2008). APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. *Dev Biol* **320**, 161-74.
Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S. and Daikuhara, Y. (1988). Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J Clin Invest* **81**, 414-9.
Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Takahashi, K., Koura, M., Hashimoto, S. and Daikuhara, Y. (1986). Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. *Exp Cell Res* **166**, 139-50.
Goll, M. G. and Bestor, T. H. (2005). Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* **74**, 481-514.
Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., Burgess, S., Haldi, M., Artzt, K., Farrington, S. et al. (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat Genet* **31**, 135-40.
Gouon-Evans, V., Boussemart, L., Gadue, P., Nierhoff, D., Koehler, C. I., Kubo, A., Shafritz, D. A. and Keller, G. (2006). BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotechnol* **24**, 1402-11.
Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J. R. and Zaret, K. S. (1996). Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev* **10**, 1670-82.
Haefliger, D. N., Moskaitis, J. E., Schoenberg, D. R. and Wahli, W. (1989). Amphibian albumins as members of the albumin, alpha-fetoprotein, vitamin D-binding protein multigene family. *J Mol Evol* **29**, 344-54.
Haffter, P. and Nusslein-Volhard, C. (1996). Large scale genetics in a small vertebrate, the zebrafish. *Int J Dev Biol* **40**, 221-7.
Harmon, E. B., Apelqvist, A. A., Smart, N. G., Gu, X., Osborne, D. H. and Kim, S. K. (2004). GDF11 modulates NGN3+ islet progenitor cell number and promotes beta-cell differentiation in pancreas development. *Development* **131**, 6163-74.
Haworth, K. E., Kotecha, S., Mohun, T. J. and Latinkic, B. V. (2008). GATA4 and GATA5 are essential for heart and liver development in Xenopus embryos. *BMC Dev Biol* **8**, 74.
Hayhurst, G. P., Strick-Marchand, H., Mulet, C., Richard, A. F., Morosan, S., Kremsdorf, D. and Weiss, M. C. (2008). Morphogenetic competence of HNF4 alpha-deficient mouse hepatic cells. *J Hepatol* **49**, 384-95.

Hentsch, B., Lyons, I., Li, R., Hartley, L., Lints, T. J., Adams, J. M. and Harvey, R. P. (1996). Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. *Genes Dev* **10**, 70-9.

Ho, C. Y., Houart, C., Wilson, S. W. and Stainier, D. Y. (1999). A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr Biol* **9**, 1131-4.

Ho, R. K. and Kimmel, C. B. (1993). Commitment of cell fate in the early zebrafish embryo. *Science* **261**, 109-11.

Holtzinger, A. and Evans, T. (2005). Gata4 regulates the formation of multiple organs. *Development* **132**, 4005-14.

Hong, S. K., Kim, C. H., Yoo, K. W., Kim, H. S., Kudoh, T., Dawid, I. B. and Huh, T. L. (2002). Isolation and expression of a novel neuron-specific onecut homeobox gene in zebrafish. *Mech Dev* **112**, 199-202.

Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D. Y. and Abdelilah-Seyfried, S. (2001). Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr Biol* **11**, 1492-502.

Horne-Badovinac, S., Rebagliati, M. and Stainier, D. Y. (2003). A cellular framework for gut-looping morphogenesis in zebrafish. *Science* **302**, 662-5.

Houssaint, E. (1980). Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. *Cell Differ* **9**, 269-79.

Huang, B. H., Laban, M., Leung, C. H., Lee, L., Lee, C. K., Salto-Tellez, M., Raju, G. C. and Hooi, S. C. (2005). Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ* **12**, 395-404.

Huang, H., Ruan, H., Aw, M. Y., Hussain, A., Guo, L., Gao, C., Qian, F., Leung, T., Song, H., Kimelman, D. et al. (2008). Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. *Development* **135**, 3209-18.

Huang, H., Vogel, S. S., Liu, N., Melton, D. A. and Lin, S. (2001). Analysis of pancreatic development in living transgenic zebrafish embryos. *Mol Cell Endocrinol* **177**, 117-24.

Hunter, M. P., Wilson, C. M., Jiang, X., Cong, R., Vasavada, H., Kaestner, K. H. and Bogue, C. W. (2007). The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev Biol* **308**, 355-67.

Hussain, S. Z., Sneddon, T., Tan, X., Micsenyi, A., Michalopoulos, G. K. and Monga, S. P. (2004). Wnt impacts growth and differentiation in ex vivo liver development. *Exp Cell Res* **292**, 157-69.

Jenuwein, T. and Allis, C. D. (2001). Translating the histone code. *Science* **293**, 1074-80.

Ju, W., Ogawa, A., Heyer, J., Nierhof, D., Yu, L., Kucherlapati, R., Shafritz, D. A. and Böttinger, E. P. (2006). Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. *Mol Cell Biol* **26**, 654-67.

Jung, J., Zheng, M., Goldfarb, M. and Zaret, K. S. (1999). Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284**, 1998-2003.

Kamiya, A., Kakinuma, S., Onodera, M., Miyajima, A. and Nakauchi, H. (2008). Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts. *Hepatology* **48**, 252-64.

Kamiya, A., Kinoshita, T. and Miyajima, A. (2001). Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS Lett* **492**, 90-4.

Keng, V. W., Fujimori, K. E., Myint, Z., Tamamaki, N., Nojyo, Y. and Noguchi, T. (1998). Expression of Hex mRNA in early murine postimplantation embryo development. *FEBS Lett* **426**, 183-6.

Keng, V. W., Yagi, H., Ikawa, M., Nagano, T., Myint, Z., Yamada, K., Tanaka, T., Sato, A., Muramatsu, I., Okabe, M. et al. (2000). Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem Biophys Res Commun* **276**, 1155-61.

Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B. and Stainier, D. Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. *Genes Dev* **15**, 1493-505.

Kinkel, M. D., Eames, S. C., Alonzo, M. R. and Prince, V. E. (2008). Cdx4 is required in the endoderm to localize the pancreas and limit beta-cell number. *Development* **135**, 919-29.

Knutson, S. K., Chyla, B. J., Amann, J. M., Bhaskara, S., Huppert, S. S. and Hiebert, S. W. (2008). Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. *EMBO J* **27**, 1017-28.

Kolterud, A., Wandzioch, E. and Carlsson, L. (2004). Lhx2 is expressed in the septum transversum mesenchyme that becomes an integral part of the liver and the formation of these cells is independent of functional Lhx2. *Gene Expr Patterns* **4**, 521-8.

Korzh, S., Emelyanov, A. and Korzh, V. (2001). Developmental analysis of ceruloplasmin gene and liver formation in zebrafish. *Mech Dev* **103**, 137-9.

Korzh, S., Pan, X., Garcia-Lecea, M., Winata, C. L., Pan, X., Wohland, T., Korzh, V. and Gong, Z. (2008). Requirement of vasculogenesis and blood circulation in late stages of liver growth in zebrafish. *BMC Dev Biol* **8**, 84.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* **128**, 693-705.

Kryukov, G. V. and Gladyshev, V. N. (2000). Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. *Genes Cells* **5**, 1049-60.

Kudoh, T., Tsang, M., Hukriede, N. A., Chen, X., Dedekian, M., Clarke, C. J., Kiang, A., Schultz, S., Epstein, J. A., Toyama, R. et al. (2001). A gene expression screen in zebrafish embryogenesis. *Genome Res* **11**, 1979-87.

Kumar, M., Jordan, N., Melton, D. and Grapin-Botton, A. (2003). Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. *Dev Biol* **259**, 109-22.

Kume, S. (2005). The molecular basis and prospects in pancreatic development. *Dev Growth Differ* **47**, 367-74.

Lagger, G., O'Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuettengruber, B., Hauser, C., Brunmeir, R., Jenuwein, T. et al. (2002). Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *EMBO J* **21**, 2672-81.

Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H. and Darnell, J. E. (1990). HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. *Genes Dev* **4**, 1427-36.

Le Dourain, N. (1975). An experimental analysis of liver development . *Med Biol* **53**.

Lee, C. S., Friedman, J. R., Fulmer, J. T. and Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature* **435**, 944-7.

Lemaigre, F. P. (2003). Development of the biliary tract. *Mech Dev* **120**, 81-7.

Lerch-Gaggl, A., Haque, J., Li, J., Ning, G., Traktman, P. and Duncan, S. A. (2002). Pescadillo is essential for nucleolar assembly, ribosome biogenesis, and mammalian cell proliferation. *J Biol Chem* **277**, 45347-55.

Leung, A. Y., Leung, J. C., Chan, L. Y., Ma, E. S., Kwan, T. T., Lai, K. N., Meng, A. and Liang, R. (2005). Proliferating cell nuclear antigen (PCNA) as a proliferative marker during embryonic and adult zebrafish hematopoiesis. *Histochem Cell Biol* **124**, 105-11.

Liao, W., Ho, C. Y., Yan, Y. L., Postlethwait, J. and Stainier, D. Y. (2000). Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development* **127**, 4303-13.

Lin, J. W., Biankin, A. V., Horb, M. E., Ghosh, B., Prasad, N. B., Yee, N. S., Pack, M. A. and Leach, S. D. (2004). Differential requirement for ptf1a in endocrine and exocrine lineages of developing zebrafish pancreas. *Dev Biol* **270**, 474-86.

Loomes, K. M., Taichman, D. B., Glover, C. L., Williams, P. T., Markowitz, J. E., Piccoli, D. A., Baldwin, H. S. and Oakey, R. J. (2002). Characterization of Notch receptor expression in the developing mammalian heart and liver. *Am J Med Genet* **112**, 181-9.

Lorent, K., Yeo, S. Y., Oda, T., Chandrasekharappa, S., Chitnis, A., Matthews, R. P. and Pack, M. (2004). Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. *Development* **131**, 5753-66.

Ma, P. and Schultz, R. M. (2008). Histone deacetylase 1 (HDAC1) regulates histone acetylation, development, and gene expression in preimplantation mouse embryos. *Dev Biol* **319**, 110-20.

Manfroid, I., Delporte, F., Baudhuin, A., Motte, P., Neumann, C. J., Voz, M. L., Martial, J. A. and Peers, B. (2007). Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. *Development* **134**, 4011-21.

Mannervik, M. and Levine, M. (1999). The Rpd3 histone deacetylase is required for segmentation of the Drosophila embryo. *Proc Natl Acad Sci USA* **96**, 6797-801.

Margagliotti, S., Clotman, F., Pierreux, C. E., Beaudry, J. B., Jacquemin, P., Rousseau, G. G. and Lemaigre, F. P. (2007). The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Dev Biol* **311**, 579-89.

Martinez Barbera, J. P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D. and Beddington, R. S. (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433-45.

Masai, I., Yamaguchi, M., Tonou-Fujimori, N., Komori, A. and Okamoto, H. (2005). The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. *Development* **132**, 1539-53.

Matsumoto, K., Yoshitomi, H., Rossant, J. and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559-63.

Matthews, R. P., Lorent, K. and Pack, M. (2008). Transcription factor onecut3 regulates intrahepatic biliary development in zebrafish. *Dev Dyn* **237**, 124-31.

Matthews, R. P., Lorent, K., Russo, P. and Pack, M. (2004). The zebrafish onecut gene hnf-6 functions in an evolutionarily conserved genetic pathway that regulates vertebrate biliary development. *Dev Biol* **274**, 245-59.

Matthews, R. P., Plumb-Rudewiez, N., Lorent, K., Gissen, P., Johnson, C. A., Lemaigre, F. and Pack, M. (2005). Zebrafish vps33b, an ortholog of the gene responsible for human arthrogryposis-renal dysfunction-cholestasis syndrome, regulates biliary development downstream of the onecut transcription factor hnf6. *Development* **132**, 5295-306.

Maurange, C., Cheng, L. and Gould, A. P. (2008). Temporal transcription factors and their targets schedule the end of neural proliferation in Drosophila. *Cell* **133**, 891-902.

Mayer, A. N. and Fishman, M. C. (2003). Nil per os encodes a conserved RNA recognition motif protein required for morphogenesis and cytodifferentiation of digestive organs in zebrafish. *Development* **130**, 3917-28.

McCright, B., Lozier, J. and Gridley, T. (2002). A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* **129**, 1075-82.

McLin, V. A., Rankin, S. A. and Zorn, A. M. (2007). Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134**, 2207-17.

Medlock, E. S. and Haar, J. L. (1983). The liver hemopoietic environment: I. Developing hepatocytes and their role in fetal hemopoiesis. *Anat Rec* **207**, 31-41.

Micsenyi, A., Tan, X., Sneddon, T., Luo, J. H., Michalopoulos, G. K. and Monga, S. P. (2004). Beta-catenin is temporally regulated during normal liver development. *Gastroenterology* **126**, 1134-46.

Milewski, W. M., Duguay, S. J., Chan, S. J. and Steiner, D. F. (1998). Conservation of PDX-1 structure, function, and expression in zebrafish. *Endocrinology* **139**, 1440-9.

Miller, D. L., Ortega, S., Bashayan, O., Basch, R. and Basilico, C. (2000). Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol Cell Biol* **20**, 2260-8.

Misra, K., Mishra, K., Gui, H. and Matise, M. P. (2008). Prox1 regulates a transitory state for interneuron neurogenesis in the spinal cord. *Dev Dyn* **237**, 393-402.

Miyajima, A., Kinoshita, T., Tanaka, M., Kamiya, A., Mukouyama, Y. and Hara, T. (2000). Role of Oncostatin M in hematopoiesis and liver development. *Cytokine Growth Factor Rev* **11**, 177-83.

Molotkov, A., Molotkova, N. and Duester, G. (2005). Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. *Dev Dyn* **232**, 950-7.

Monga, S. P., Mars, W. M., Pediaditakis, P., Bell, A., Mulé, K., Bowen, W. C., Wang, X., Zarnegar, R. and Michalopoulos, G. K. (2002). Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. *Cancer Res* **62**, 2064-71.

Monga, S. P., Monga, H. K., Tan, X., Mulé, K., Pediaditakis, P. and Michalopoulos, G. K. (2003). Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology* **124**, 202-16.

Mudumana, S. P., Wan, H., Singh, M., Korzh, V. and Gong, Z. (2004). Expression analyses of zebrafish transferrin, ifabp, and elastaseB mRNAs as differentiation markers for the three major endodermal organs: liver, intestine, and exocrine pancreas. *Dev Dyn* **230**, 165-73.

Müller, M., v Weizsäcker, E. and Campos-Ortega, J. A. (1996). Expression domains of a zebrafish homologue of the Drosophila pair-rule gene hairy correspond to primordia of alternating somites. *Development* **122**, 2071-8.

Nadauld, L. D., Shelton, D. N., Chidester, S., Yost, H. J. and Jones, D. A. (2005). The zebrafish retinol dehydrogenase, rdh1l, is essential for intestinal development and is regulated by the tumor suppressor adenomatous polyposis coli. *J Biol Chem* **280**, 30490-5.

Nambiar, R. M. and Henion, P. D. (2004). Sequential antagonism of early and late Wnt-signaling by zebrafish colgate promotes dorsal and anterior fates. *Dev Biol* **267**, 165-80.

Nambiar, R. M., Ignatius, M. S. and Henion, P. D. (2007). Zebrafish colgate/hdac1 functions in the non-canonical Wnt pathway during axial extension and in Wnt-independent branchiomotor neuron migration. *Mech Dev* **124**, 682-98.

Ng, A. N., de Jong-Curtain, T. A., Mawdsley, D. J., White, S. J., Shin, J., Appel, B., Dong, P. D., Stainier, D. Y. and Heath, J. K. (2005). Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. *Dev Biol* **286**, 114-35.

Nguyen, T. T., Cho, K., Stratton, S. A. and Barton, M. C. (2005). Transcription factor interactions and chromatin modifications associated with p53-mediated, developmental repression of the alpha-fetoprotein gene. *Mol Cell Biol* **25**, 2147-57.

Nusslein-Volhard and Dahm, R. (2002). Zebrafish. *Oxford University Press*.

Ober, E. A., Field, H. A. and Stainier, D. Y. (2003). From endoderm formation to liver and pancreas development in zebrafish. *Mech Dev* **120**, 5-18.

Ober, E. A., Verkade, H., Field, H. A. and Stainier, D. Y. (2006). Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* **442**, 688-91.

Odenthal, J. and Nüsslein-Volhard, C. (1998). fork head domain genes in zebrafish. *Dev Genes Evol* **208**, 245-58.

Odom, D. T., Zizlsperger, N., Gordon, D. B., Bell, G. W., Rinaldi, N. J., Murray, H. L., Volkert, T. L., Schreiber, J., Rolfe, P. A., Gifford, D. K. et al. (2004). Control of pancreas and liver gene expression by HNF transcription factors. *Science* **303**, 1378-81.

Oström, M., Loffler, K. A., Edfalk, S., Selander, L., Dahl, U., Ricordi, C., Jeon, J., Correa-Medina, M., Diez, J. and Edlund, H. (2008). Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. *PLoS ONE* **3**, e2841.

Papoutsi, M., Dudas, J., Becker, J., Tripodi, M., Opitz, L., Ramadori, G. and Wilting, J. (2007). Gene regulation by homeobox transcription factor Prox1 in murine hepatoblasts. *Cell Tissue Res* **330**, 209-20.

Park, H. C., Boyce, J., Shin, J. and Appel, B. (2005). Oligodendrocyte specification in zebrafish requires notch-regulated cyclin-dependent kinase inhibitor function. *J Neurosci* **25**, 6836-44.

Parviz, F., Matullo, C., Garrison, W. D., Savatski, L., Adamson, J. W., Ning, G., Kaestner, K. H., Rossi, J. M., Zaret, K. S. and Duncan, S. A. (2003). Hepatocyte

nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* **34**, 292-6.

Pelton, R. W., Saxena, B., Jones, M., Moses, H. L. and Gold, L. I. (1991). Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *J Cell Biol* **115**, 1091-105.

Pierreux, C. E., Vanhorenbeeck, V., Jacquemin, P., Lemaigre, F. P. and Rousseau, G. G. (2004). The transcription factor hepatocyte nuclear factor-6/Onecut-1 controls the expression of its paralog Onecut-3 in developing mouse endoderm. *J Biol Chem* **279**, 51298-304.

Pillai, R., Coverdale, L. E., Dubey, G. and Martin, C. C. (2004). Histone deacetylase 1 (HDAC-1) required for the normal formation of craniofacial cartilage and pectoral fins of the zebrafish. *Dev Dyn* **231**, 647-54.

Plaster, N., Sonntag, C., Schilling, T. F. and Hammerschmidt, M. (2007). REREa/Atrophin-2 interacts with histone deacetylase and Fgf8 signaling to regulate multiple processes of zebrafish development. *Dev Dyn* **236**, 1891-904.

Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D. et al. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* **124**, 2935-44.

Rai, K., Chidester, S., Zavala, C. V., Manos, E. J., James, S. R., Karpf, A. R., Jones, D. A. and Cairns, B. R. (2007). Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. *Genes Dev* **21**, 261-6.

Rai, K., Nadauld, L. D., Chidester, S., Manos, E. J., James, S. R., Karpf, A. R., Cairns, B. R. and Jones, D. A. (2006). Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. *Mol Cell Biol* **26**, 7077-85.

Reiter, J. F., Kikuchi, Y. and Stainier, D. Y. (2001). Multiple roles for Gata5 in zebrafish endoderm formation. *Development* **128**, 125-35.

Rodriguez, T. A., Casey, E. S., Harland, R. M., Smith, J. C. and Beddington, R. S. (2001). Distinct enhancer elements control Hex expression during gastrulation and early organogenesis. *Dev Biol* **234**, 304-16.

Rojas, A., De Val, S., Heidt, A. B., Xu, S. M., Bristow, J. and Black, B. L. (2005). Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. *Development* **132**, 3405-17.

Rossi, J. M., Dunn, N. R., Hogan, B. L. and Zaret, K. S. (2001). Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev* **15**, 1998-2009.

Roy, S., Qiao, T., Wolff, C. and Ingham, P. W. (2001). Hedgehog signaling pathway is essential for pancreas specification in the zebrafish embryo. *Curr Biol* **11**, 1358-63.

Sadler, K. C., Krahn, K. N., Gaur, N. A. and Ukomadu, C. (2007). Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proc Natl Acad Sci USA* **104**, 1570-5.

Sagerström, C. G., Kao, B. A., Lane, M. E. and Sive, H. (2001). Isolation and characterization of posteriorly restricted genes in the zebrafish gastrula. *Dev Dyn* **220**, 402-8.

Sakaguchi, T. F., Sadler, K. C., Crosnier, C. and Stainier, D. Y. (2008). Endothelial signals modulate hepatocyte apicobasal polarization in zebrafish. *Curr Biol* **18**, 1565-71.

Saleh, M., Rambaldi, I., Yang, X. J. and Featherstone, M. S. (2000). Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. *Mol Cell Biol* **20**, 8623-33.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E. and Birchmeier, C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**, 699-702.

Schuettengruber, B., Simboeck, E., Khier, H. and Seiser, C. (2003). Autoregulation of mouse histone deacetylase 1 expression. *Mol Cell Biol* **23**, 6993-7004.

Sekhon, S. S., Tan, X., Micsenyi, A., Bowen, W. C. and Monga, S. P. (2004). Fibroblast growth factor enriches the embryonic liver cultures for hepatic progenitors. *Am J Pathol* **164**, 2229-40.

Shin, D., Shin, C. H., Tucker, J., Ober, E. A., Rentzsch, F., Poss, K. D., Hammerschmidt, M., Mullins, M. C. and Stainier, D. Y. (2007). Bmp and Fgf signaling are essential for liver specification in zebrafish. *Development* **134**, 2041-50.

Sladek, F. M., Zhong, W. M., Lai, E. and Darnell, J. E. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev* **4**, 2353-65.

Song, J., Kim, H. J., Gong, Z., Liu, N. A. and Lin, S. (2007). Vhnf1 acts downstream of Bmp, Fgf, and RA signals to regulate endocrine beta cell development in zebrafish. *Dev Biol* **303**, 561-75.

Sosa-Pineda, B., Wigle, J. T. and Oliver, G. (2000). Hepatocyte migration during liver development requires Prox1. *Nat Genet* **25**, 254-5.

Späth, G. F. and Weiss, M. C. (1998). Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells. *J Cell Biol* **140**, 935-46.

Stadler, J. A., Shkumatava, A., Norton, W. H., Rau, M. J., Geisler, R., Fischer, S. and Neumann, C. J. (2005). Histone deacetylase 1 is required for cell cycle exit and differentiation in the zebrafish retina. *Dev Dyn* **233**, 883-9.

Stafford, D. and Prince, V. E. (2002). Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. *Curr Biol* **12**, 1215-20.

Stafford, D., White, R. J., Kinkel, M. D., Linville, A., Schilling, T. F. and Prince, V. E. (2006). Retinoids signal directly to zebrafish endoderm to specify insulin-expressing beta-cells. *Development* **133**, 949-56.

Stenvers, K. L., Tursky, M. L., Harder, K. W., Kountouri, N., Amatayakul-Chantler, S., Grail, D., Small, C., Weinberg, R. A., Sizeland, A. M. and Zhu, H. J. (2003). Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol Cell Biol* **23**, 4371-85.

Suksaweang, S., Lin, C. M., Jiang, T. X., Hughes, M. W., Widelitz, R. B. and Chuong, C. M. (2004). Morphogenesis of chicken liver: identification of localized growth zones and the role of beta-catenin/Wnt in size regulation. *Dev Biol* **266**, 109-22.

Sumazaki, R., Shiojiri, N., Isoyama, S., Masu, M., Keino-Masu, K., Osawa, M., Nakauchi, H., Kageyama, R. and Matsui, A. (2004). Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. *Nat Genet* **36**, 83-7.

Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H. and Taniguchi, H. (2003). Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**, 2513-24.

Tan, X., Yuan, Y., Zeng, G., Apte, U., Thompson, M. D., Cieply, B., Stolz, D. B., Michalopoulos, G. K., Kaestner, K. H. and Monga, S. P. (2008). Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. *Hepatology* **47**, 1667-79.

Tanaka, H., Yamamoto, T., Ban, T., Satoh, S., Tanaka, T., Shimoda, M., Miyazaki, J. and Noguchi, T. (2005). Hex stimulates the hepatocyte nuclear factor 1alpha-mediated activation of transcription. *Arch Biochem Biophys* **442**, 117-24.

Tang, Y., Katuri, V., Dillner, A., Mishra, B., Deng, C. X. and Mishra, L. (2003). Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. *Science* **299**, 574-7.

Tanimizu, N. and Miyajima, A. (2004). Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *J Cell Sci* **117**, 3165-74.

Thisse, C., Degrave, A., Kryukov, G. V., Gladyshev, V. N., Obrecht-Pflumio, S., Krol, A., Thisse, B. and Lescure, A. (2003). Spatial and temporal expression patterns of selenoprotein genes during embryogenesis in zebrafish. *Gene Expr Patterns* **3**, 525-32.

Thompson, M. D. and Monga, S. P. (2007). WNT/beta-catenin signaling in liver health and disease. *Hepatology* **45**, 1298-305.

Torres-Padilla, M. E., Sladek, F. M. and Weiss, M. C. (2002). Developmentally regulated N-terminal variants of the nuclear receptor hepatocyte nuclear factor 4alpha mediate multiple interactions through coactivator and corepressor-histone deacetylase complexes. *J Biol Chem* **277**, 44677-87.

Tou, L., Liu, Q. and Shivdasani, R. A. (2004). Regulation of mammalian epithelial differentiation and intestine development by class I histone deacetylases. *Mol Cell Biol* **24**, 3132-9.

Wallace, K. N., Yusuff, S., Sonntag, J. M., Chin, A. J. and Pack, M. (2001). Zebrafish hhex regulates liver development and digestive organ chirality. *Genesis* **30**, 141-3.

Wang, A. G., Kim, S. U., Lee, S. H., Kim, S. K., Seo, S. B., Yu, D. Y. and Lee, D. S. (2005). Histone deacetylase 1 contributes to cell cycle and apoptosis. *Biol Pharm Bull* **28**, 1966-70.

Wang, G. L., Salisbury, E., Shi, X., Timchenko, L., Medrano, E. E. and Timchenko, N. A. (2008a). HDAC1 cooperates with C/EBPalpha in the inhibition of liver proliferation in old mice. *J Biol Chem* **283**, 26169-78.

Wang, G. L., Salisbury, E., Shi, X., Timchenko, L., Medrano, E. E. and Timchenko, N. A. (2008b). HDAC1 promotes liver proliferation in young mice via interactions with C/EBPbeta. *J Biol Chem* **283**, 26179-87.

Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R. and Darlington, G. J. (1995). Impaired energy homeostasis in C/EBP alpha knockout mice. *Science* **269**, 1108-12.

Warga, R. M. and Nüsslein-Volhard, C. (1999). Origin and development of the zebrafish endoderm. *Development* **126**, 827-38.

Watt, A. J., Zhao, R., Li, J. and Duncan, S. A. (2007). Development of the mammalian liver and ventral pancreas is dependent on GATA4. *BMC Dev Biol* **7**, 37.

Weidner, K. M., Arakaki, N., Hartmann, G., Vandekerckhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T. and Daikuhara, Y. (1991).

Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc Natl Acad Sci USA* **88**, 7001-5.

Weinstein, M., Monga, S. P., Liu, Y., Brodie, S. G., Tang, Y., Li, C., Mishra, L. and Deng, C. X. (2001). Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. *Mol Cell Biol* **21**, 5122-31.

Westendorf, J. J. (2007). Histone deacetylases in control of skeletogenesis. *J Cell Biochem* **102**, 332-40.

Westerfield, M. (2000). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). *University of Oregon Press*.

Whetstine, J. R., Ceron, J., Ladd, B., Dufourcq, P., Reinke, V. and Shi, Y. (2005). Regulation of tissue-specific and extracellular matrix-related genes by a class I histone deacetylase. *Mol Cell* **18**, 483-90.

Wilson, A. J., Byun, D. S., Popova, N., Murray, L. B., L'Italien, K., Sowa, Y., Arango, D., Velcich, A., Augenlicht, L. H. and Mariadason, J. M. (2006). Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* **281**, 13548-58.

Xu, R. H., Peng, Y., Fan, J., Yan, D., Yamagoe, S., Princler, G., Sredni, D., Ozato, K. and Kung, H. F. (2000). Histone acetylation is a checkpoint in FGF-stimulated mesoderm induction. *Dev Dyn* **218**, 628-35.

Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H. and Masai, I. (2005). Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. *Development* **132**, 3027-43.

Yamasaki, H., Sada, A., Iwata, T., Niwa, T., Tomizawa, M., Xanthopoulos, K. G., Koike, T. and Shiojiri, N. (2006). Suppression of C/EBPalpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. *Development* **133**, 4233-43.

Yanai, M., Tatsumi, N., Hasunuma, N., Katsu, K., Endo, F. and Yokouchi, Y. (2008). FGF signaling segregates biliary cell-lineage from chick hepatoblasts cooperatively with BMP4 and ECM components in vitro. *Dev Dyn* **237**, 1268-83.

Yarden, A. and Geiger, B. (1996). Zebrafish cyclin E regulation during early embryogenesis. *Dev Dyn* **206**, 1-11.

Yatskievych, T. A., Pascoe, S. and Antin, P. B. (1999). Expression of the homebox gene Hex during early stages of chick embryo development. *Mech Dev* **80**, 107-9.

Yelon, D., Horne, S. A. and Stainier, D. Y. (1999). Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev Biol* **214**, 23-37.

Yelon, D., Ticho, B., Halpern, M. E., Ruvinsky, I., Ho, R. K., Silver, L. M. and Stainier, D. Y. (2000). The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. *Development* **127**, 2573-82.

Yoshida, M., Horinouchi, S. and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *Bioessays* **17**, 423-30.

Zecchin, E., Mavropoulos, A., Devos, N., Filippi, A., Tiso, N., Meyer, D., Peers, B., Bortolussi, M. and Argenton, F. (2004). Evolutionary conserved role of ptf1a in the specification of exocrine pancreatic fates. *Dev Biol* **268**, 174-84.

Zhang, W., Yatskievych, T. A., Baker, R. K. and Antin, P. B. (2004). Regulation of Hex gene expression and initial stages of avian hepatogenesis by Bmp and Fgf signaling. *Dev Biol* **268**, 312-26.

Zhang, W., Yatskievych, T. A., Cao, X. and Antin, P. B. (2002). Regulation of Hex gene expression by a Smads-dependent signaling pathway. *J Biol Chem* **277**, 45435-41.

Zhao, R., Watt, A. J., Li, J., Luebke-Wheeler, J., Morrisey, E. E. and Duncan, S. A. (2005). GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. *Mol Cell Biol* **25**, 2622-31.